DISTRIBUTION, ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS, WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCUS

Dissertation submitted for

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MAY 2018

CERTIFICATE

This is to certify that this dissertation entitled **"DISTRIBUTION, ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS, WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCUS"** is the bonafide original work done by **Dr.R.VENNILA,** Post graduate in Microbiology, during the period of April 2016 to March 2017 under the guidance of **Prof.Dr.UMADEVI.U.M.D.,** Professor of Microbiology, Institute of Microbiology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai-600003, in partial fulfilment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV) held in May 2018.**

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DECLARATION

I, **Dr.R.Vennila,** Post Graduate, Institute of Microbiology, Madras Medical college, solemnly declare that the dissertation titled **"DISTRIBUTION, ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS, WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCUS"** is the bonafide work done by me at the Institute of Microbiology, Madras Medical College, Chennai, under the guidance and supervision of **Dr.UMADEVI.U.MD.,** Professor, Institute of Microbiology, Madras Medical College, Chennai-600 003. The dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfilment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in May 2018.

Place: Chennai,

Date: **Dr. R.VENNILA**

Signature of the Guide

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Introduction

INTRODUCTION

Enterococci are commensal microorganisms that act as potential human pathogens, causing a variety of infections in humans. Over the last decades, they have been emerged from long and being considered virtually harmless bacteria to medically important multiple–antibiotic resistant nosocomial pathogens that contribute significantly to patient morbidity and mortality, as well as health care costs.[1] *Enterococcus* species have been recognized as a nosocomial pathogens causing diseases like bacteremia, endocarditis, complicated urinary tract infections, intraabdominal infections, pelvic infections, wound and soft tissue infections, neonatal sepsis and rarely meningitis.[2] Enterococcal bacteremia is frequently associated with metastatic abcesses in multiple organs and high mortality rates.

- In critically ill patients infections caused by antibiotic resistant *Enterococci* pose therapeutic challenge.[3] They are becoming increasingly important agents of human disease, largely because of their resistance to antimicrobial agents. Hence it is essential to determine the distribution and antimicrobial susceptibility pattern of *Enterococci*. They show intrinsic resistance to a number of commonly used antibiotics like penicillin, cephalosporin and low level amino glycosides, acquired resistance to glycopeptides like vancomycin & teicoplanin antibiotics either by mutation or through transfer of mobile genetic elements carrying resistance

genes/ virulence factors.[4] Drug of choice for aminoglycoside resistant *Enterococci* is vancomycin.[5]

- Vancomycin Resistant *Enterococci* (VRE) pose an emerging problem in hospitals worldwide because of widespread use of vancomycin and extended spectrum cephalosporins. Vancomycin resistant *Enterococcus* infections are serious because the last option for treatment of life threatening infections are glycopeptides, therefore it leads to increase in mortality rate. *Enterococcus faecalis* and *Enterococcus faecium* account for majority of vancomycin resistant *Enterococcus* infections. The Vancomycin resistance of *Enterococcus* is mediated by a group of genes like van A, van B, van C, van D and van E. These genes are transcribed in presence of vancomycin & low affinity cell wall precursors to vancomycin are synthesized.[4] Horizontal gene transfer is mainly responsible for transfer of virulence genes & spread of resistance.[4]

- The ability to form biofilm has recently been listed among the most prominent virulence properties of these microorganisms, allowing colonization of inert and biological surfaces while protecting against antimicrobial substances and mediating adhesion and invasion of host cells. Biofilm formation may be of particular importance in the development of endocarditis, endodontic and urinary infections and medical implant device associated infections.[1] *Enterococci* isolates which

produce biofilm are highly resistant to antibiotics than non-biofilm producers.[2]

- Vancomycin resistant *Enterococcus* infection rates are highest among critically ill patients in ICUs who have limited treatment options.[6] VRE infections remain a challenge with 60% to 70 % mortality rate and has poor outcome.[6] In recent days there is increasing rate of VRE infections and colonization and their clinical outcomes.[6] The alarming thing is risk of transfer of vancomycin resistance gene from VRE to some gram positive organisms like staphylococcus aureus through conjugate plasmids, which worsens sceinerio further.[7] Hence it is important to monitor continuously the infections caused by VRE and their antimicrobial susceptibility pattern. Prevalence and changing trends of VRE infections are assessed and it shows immense help in planning for infection control measures. This should be implemented in the hospital and also in the community to reduce the mortality and morbidity caused by these VRE infections. Aim of the study was to determine the prevalence of vancomycin resistant *Enterococci* in a tertiary care hospital and to determine the distribution of genes among them. In addition an attempt was made to analyse on associated risk factors & biofilm formation among them.

Aim and objectives

AIM & OBJECTIVES

AIM :

To study the prevalence of vancomycin resistance among the *Enterococcal* species isolated from various clinical samples such as urine, blood, pus, tissue fluids and feces obtained from the patients of a tertiary care hospital.

OBJECTIVES:

- To isolate and characterize phenotypically *Enterococcus species* from various clinical specimens.
- To study antimicrobial susceptibility pattern of *Enterococci* by Kirby Bauer Disk diffusion method.
- To study about the detection of biofilm formation in *Enterococcus species* by Microtitre plate method.
- To determine Minimum inhibitory concentration of vancomycin by Microbroth dilution method.
- Phenotypic and genotypic characterization of vancomycin resistant *Enterococci* by molecular methods.

Review of literature

REVIEW OF LITERATURE

HISTORICAL PERSPECTIVES

Theircelin in 1899 used the term "*enterocoque"* in a french publication to describe a bacteria seen in pairs and short chains in human feces. This was later included in new genus *Enterococcus* proteiforms**.**[3] Later MacCallum and Hastings in the same year had described a fatal case of endocarditis from the John hopkins hospital caused by a bacterium that was very hard and tenacious of life which they termed as "*Micrococcus zymogenes*" was later called as *S.faecalis* var zymogenes**.**[3] They confirmed the pathogenicity of new organism by satisfying koch's postulates. Andrews and Horder first coined the name *Streptococcus faecalis* in 1906, for an isolate recovered from the blood of a patient with endocarditis, which is so characteristic with the organism identified in human intestine.[8] Later in 1918, Orla-Jensen described *Streptococcus faecium*, a second organism of this group.[8] In 1935, Sherman and Wing, proposed a third species *Streptococcus durans*. In 1937 Sherman emphasised the term *Enterococcus* **.**[8] *Enterococci* was originally classified as *Streptococci* because the two genera share many morphological and phenotypic characters. In 1967, Nowlan and Diebel added *Streptococcus* avium to the *Enterococcus* group. In 1970, a formal proposed by Kalina that *Enterococcal Streptococci* be considered a new genus was put forward based on their distinct phenotypic characteristics but were not officially recognized.

The members of the Genus *Enterococcus* are gram positive cocci that occur singly or arranged in pairs and short chains, catalase negative (except E.haemoperoxidus),few strains produce weak effervescence when grown on blood agar. They are nonmotile (except *E.gallinarum* and *E.casseliflavus*) and noncapsulated.[9] *Enterococci* are facultative anaerobes that follows Embden Meyerhof pathway where glucose is fermented and lactic acid is produced as an end product. *Enterococci* are referred as typical lactic acid bacteria.[10] *Enterococci* are anaerogenic, grow at temperatures between 10 to 45°c, grow in 6.5%Nacl, grow at PH 9.6, survive at 60°c for 30 minutes and hydrolyse esculin in the presence of bile salts.[5] Many of these characteristics are used to distinguish *Enterococci* from nonenterococcal *streptococci*. In 1984 Schleifer and Kilpper-Balz provided genetic evidence used DNA-DNA and DNA-rRNA hybridization studies to prove that *E.faecalis* and *E.faecium* were different from other members of genus streptococcus and suggested to provide a separate genus[8]. The G+C content of DNA ranges from 32-44mol% and genomic size were 2000-3500 kb.[8]

Enterococci are usually alpha hemolytic or non hemolytic that show inability to lyse bovine RBCs which are commonly used in agar plates, however some *E.faecalis* do lyse RBCs (beta hemolysis) from human, horses and rabbits.[3] On blood agar after 24 hours growth, they produce white to grey coloured colonies,1-2mm diameter and on Macconkey agar 0.5-1mm majenta pink coloured colonies are produced. Most of the species hydrolyze L-pyrollidonyl-beta-naphthylamide by producing pyrollidonyl arylamidase (PYRase)(except for *E.cecorum*, *E. columbae*, *E. pallens*, and *E. saccharolyticus*).[8] All strains

hydrolyze leucine β naphthylamide by producing leucine aminopeptidase (LAPase**).**[3] *Enterococci* do not synthesis porphyrin and so do not express cytochrome enzymes.[11] Cytochrome oxidase activity is expressed when *E.faecalis* are grown in blood containing media.

Some species produce yellowish pigment E.mundii, *E.casseliflavus*, E.sulfureus , E.pallens & E.gilvus. Around 37 species have been identified from clinical isolates, the predominant is *E.faecalis (80-90%) & E.faecium*(5-10%).Other species like *E.gallinarum, E.durans, E.hirae, E.avium, E.dispar*, *E.malodoratusare* less frequently identified.[3] The *Enterococcus* reacted with group D antisera, while the pyogenic *streptococci* reacted with group A,B,C,E,F,or G and the viridans *streptococci* were nongroupable. A cell wall associated glycerol teichoic acid antigen called Lancefield's group D antigen is produced by most strains.[3] *Enterococci* were classified as group D *streptococci* on the basis of their colony morphology and reaction with group specific antisera.

HABITAT

Enterococci are found in the faeces of most healthy adults. They are numerous in the large intestine where concentrations of 10^5 to 10^7 bacteria per gram. Due to several intrinsic characteristics they grow and survive in harsh environments. *Enterococci* is widespread in soil, plants, water, food and animals including mammals, birds, insects and reptiles. In humans, major habitat of *Enterococci* appears to be the gastrointestinal tract although less frequently from other sites such as vagina, anterior urethra, skin, oropharynx and bile ducts**.**[1] The

term "*fecal Enterococci*" includes four species: *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae*. They are considered to be good indicators of fecal contaminant of food and water since they are present in the feces of humans & warm-blooded animals. The prevalence of *Enterococcus* species differs according to host factors, underlying conditions & prior antimicrobial therapy.[12] Long term hemodialysis and cardiac patients had higher rates of *Enterococcus* carriage than acute dental patients. Apart from in-vitro transfer, in-vivo transfer of antibiotic resistance genes under natural conditions between *Enterococcus faecalis* in sewage water treatment plants has been described and it demonstrates the role of environmental strains in the dissemination of antibiotic resistance.

EPIDEMIOLOGY

A nosocomial infection is one for which there is no evidence that the infection was present or incubating at the time of hospital admission & it is one of the major health problem globally. *Enterococci* are among the leading causes of hospital acquired infections in the United States (second to third), and it is estimated that in 2004, 521,285 hospital discharges were associated with Enterococcal infections.[13] The emergence of drug resistance among many bacteria has raised since hospitals serve to be the ideal ground for the development and spread of several multi-drug resistant bacteria. Over the past decade there is a shift in prevalence from gram-negative to gram-positive species as predominant cause of nosocomial infections, among which *Enterococci* has become one of the top three pathogens causing various infections like urinary tract infections (UTI), blood stream infections (BSI),skin and soft tissue infections (SSTI) and other miscellaneous infections.

The predominant species encountered in nosocomial infections is *E.faecalis,* however in the past few decades *E.faecium* is on the rise. Now-a-days *E.faecium* is isolated as common as *E.faecalis* in hospital associated infections. About 30% of Enterococcal infections are caused by VRE, among which *E.faecium* being the commonest (>90%)**.**[14] The first step in infectious process is colonization of the gastrointestinal tract by hospital-associated strains. Risk factors for increased VRE colonization include immunosuppression, serious comorbid conditions (such as diabetes, renal failure), increased hospital stay, residence in a long-term care facility, hospitalization in a room previously

occupied by a patient colonized with VRE, invasive procedures and administration of broad-spectrum antibiotics (e.g., cephalosporins), or vancomycin.[6,4] Several epidemiological studies conducted in human subjects from community showed *Enterococci* are resistant to various antimicrobials like ampicillin, gentamicin and vancomycin. Most of the studies have concluded that previous hospitalization and prior use of vancomycin are common factors for community dissemination of vancomycin resistant *Enterococcus* (VRE).

VIRULENCE FACTORS

The virulence of *Enterococci* is regulated by virulence coding genes present in the pathogenic islands (PAI) of the genome. The PAI of *Enterococcus* was first identified in the genome of multidrug resistant strains of *E.faecalis* that caused nosocomial infections in 1980s.[15] The ability of the organism to acquire new traits and colonize new areas enhance virulence.

HEMOLYSIN

Cytolysin /hemolysin- a heterodimeric toxin, pheromone encoded plasmid secreted by certain strains of *E.faecalis* lyses human, rabbit, equine and bovine erythrocytes (but not sheep RBCs) and polymorphonuclear leucocytes and macrophages.[16] It produces significant toxicity in endocarditis and rabbit endophthalmitis models. Hemolysin producing strains cause more severe infections & is regulated by a novel, two component regulatory system via quorum sensing.[8]

GELATINASE & SERINE PROTEASE

Proteases produced by *Enterococci* have the capacity to hydrolyze gelatin, collagen, casein and other peptides. *E.faecalis* protease Gel E also modifies the critical components of immune system. The expression of gelatinase is regulated by frc locus in *E.faecalis*. The expression of gelatinase and serine protease genes is regulated by *fsr* system, which is a two component quorum-sensing regulatory system that regulates the expression of multiple genes in *E. faecalis* and is similar to the *agr* system of *Staphylococcus aureus.* [17,18]. Mutants lacking the genes corresponding to this protein are highly attenuated in experimental endocarditis, peritonitis and endophthalmitis.

ENTEROCOCCAL SURFACE PROTEIN(ESP)

Cell wall-associated protein of *E. faecalis* called Enterococcal surface protein- ESP acts as an adhesin like aggregation substance. These adhesins would contribute as bacterial extracellular matrix molecules in humans. ESP variants, were reported in E. *faecium* clinical isolates. ESP contributes to colonization and persistence of *E.faecalis* in the urinary bladder wall through specific components such as mucin or uroplakin. Later studies have shown that ESP was not required, but its presence was associated with higher amounts of biofilm .[8]

AGGREGATION SUBSTANCE(AS)

Aggregation substance is a pheromone inducible surface protein which promotes adherence of *Enterococci* to intestinal & renal epithelial cells and clumping of organisms facilitating plasmid exchange. In vivo aggregation

substance contributes to the pathogenesis of Enterococcal infection by exhibiting resistance to killing by polymorphonuclear leukocytes and macrophages, thereby promoting intracellular survival inside macrophages. AS also contributes to adherence of Enterococcal isolates to host tissues.[19]

MSCRAMM Ace

The surface proteins – Ace (adhesion of collagen of *E.faecalis*) and Acm (homologue adhesion of *E.faecium)* are microbial surface components recognizing adhesive matrix molecules (MSCRAMM) structurally and functionally related to staphylococcal CNA adhesion factor.[20] They are involved in attachment of bacteria to host proteins like collagen, fibrinogen and fibronectin. Similar to MSCRAMM other surface proteins playing role in virulence, are second collagen adhesion of *E.faecium* (Scm), Enterococcal surface protein (Espfc) of *E.faecalis* and of *E.faecium* (Espfm), surface proteins (Fms) of *E.faecium*, SgrA (which binds to basal lamina components), EcbA (binds to collagen type V).[3]

CAPSULAR POLYSACCHARIDE

Capsular polysaccharides (on bacterial cell surface) contribute to the pathogenicity by interfering with phagocytosis by host immune cells. An operon encodes the synthesis of capsular polysaccharide in most clinical isolates of *E.faecalis. The* capsular polysaccharides are also the potential targets of immunotherapy.[8]

LIPOTEICHOIC ACID

Lipoteichoic acid constitute the group D antigen of *Enterococci,* functions in modulating immune response by inducing the production of TNF and Interferons.

COCCOLYSIN

Coccolysin, an extracellular metallo-endopeptidase secreted by some *E.faealis* strains mediate virulence by inactivating endothelin, the vasoactive peptide .

EXTRACELLULAR SUPEROXIDE

Extracellular superoxide, secreted in large amounts by most of *E.faecalis and E.faecium* strains enhances in vivo survival in mixed infection with *Bacteroids fragilis* in a subcutaneous infections.[21] *E.faecalis* isolates from blood are unique to produce superoxide.

HYARULANIDASE

Hyarulanidase is a cell surface associated enzyme, clearing the mucopolysaccharide moiety of connective tissue cartilage. Detection of hyarulanidase is accomplished by the inoculation of semisolid media with hyarulanic acid. This enzyme contributes to virulence of the *Enterococcus* isolates.

PHREMONES

They are the small peptides secreted by *E.faecalis.* It promotes conjugative transfer of plasmid DNA between strains. They are chromosomally encoded & elicit a specific mating response from plasmid carrying donor cells. A few phremones act as chemoattractant for neutrophils.

OTHER VIRULENCE FACTORS

- AS-48 is a plasmid coded peptide produced by *E.faecalis* that inhibits both Gram positive and Gram negative bacteria. It causes lysis by pore generation in cytoplasmic membrane of target cells leading to depolarization and it induces lysis of some *Enterococcus* by activating autolysin.
- Pili is present in both *E.faecalis* and *E.faecium* which mediates attachment and invasion into host tissues.
- *E.faecalis* stress protein Gls24 responsible for Enterococcal resistance to bile salts is important in pathogenicity of endocarditis .
- *hyl Efm*- containing plasmids found in *E.faecium*, increase the colonizing capacity.[3]

PATHOGENESIS

The critical factor in the pathogenesis of Enterococcal infection is the higher level of gastro intestinal colonization. *Enterococci* are minor population, where anaerobes are predominant in a normal host and have symbiotic relationship between host immune system and with other bacteria. One of the

important factor that increases gastro intestinal colonization of *Enterococci* (eg.VRE) is the administration of antimicrobial agents that are excreted in bile or having anti anaerobic activity without disturbing *Enterococci* (eg, various cephalosporins).[21] Administration of broad-spectrum antibiotics favours down regulation of the intestinal expression of RegIIIγ (a bactericidal lectin produced by intestinal epithelial cells and Paneth cells), which is activity against Gram positive intestinal organisms thereby favours VRE colonization of the gastrointestinal tract . VRE colonization is also influenced by some gut anaerobic microbiota (Barnesiella species in mice) and increased stomach pH. Once *Enterococci* have established their niche, they gain access to blood stream and lymphatics through the mechanism which is poorly understood. The intrinsic resistance to the commonly used antibiotics, pathogenecity island, virulence associated genes, multi-drug resistance etc… allow the bacterium to overcome host defense mechanisms. Along with these, the changed dynamics of host – commensal relationships like diminished host immunity, injury to the host and broad spectrum antibiotic use, promote colonization, favour enterococcal infections and its dissemination to the environment and to other tissues.[1]Thus the virulent strains exhibit survival advantage over others and persist for a longer period of time in the environment and host and play as one of the predominent nosocomial pathogens. One of the interesting factor that *Enterococci* can transfer the resistant genes to other Gram positive bacteria[14,8] would further increase their clinical significance.

CLINICAL SYNDROMES

1. URINARY TRACT INFECTIONS

Enterococci are well known causative agents of nosocomial UTIs, associated with anatomic abnormalities of genitourinary tract, instrumentation, indwelling catheters, prior antibiotic use and recurrent UTIs. The centres for disease control (CDC) National Nosocomial surveillance study (NNSS) states *Enterococci* being the third most common cause of catheter associated UTIs. Among them *E.faecium* being the predominant species (40%) followed by *E.faecalis* (25%) and other species (35%). It is very difficult in the hospital setting to differentiate infection from colonization. The factors that help to differentiate are presence of leucocytes in urine along with fever, local symptoms and signs, systemic manifestations and a colony count of > 10^5CFU/ml.[3,8] Removal of catheter itself helps to eradicate the organism. *Enterococcus* can cause complicated UTIs also which lead to complications like are pyelnephritis, perinephric abcess and recurrent bacteremic episodes.[3,8]

RISK FACTORS FOR ENTEROCOCCAL UTIs

1. Indwelling catheterization
2. Instrumentation
3. Anatomic abnormalities of genitourinary tract
4. Elderly patients
5. Chronic debilitated patients
6. Prolonged hospitalisation

2. INTRA ABDOMINAL AND PELVIC INFECTIONS

Enterococci being the second most common isolate from abdominal pelvic infections along with Gram negative and anaerobic organisms. *Enterococci* produce spontaneous peritonitis and empyema in patients with cirrhosis, acute renal failure and continuous ambulatory peritonitis.[9] Presence of *Enterococcus* in intraabdominal and pelvic infections indicates treatment failure, increases postoperative complications and mortality.[22] Because of emergence of vancomycin resistant *Enterococcus* and increase in the multidrug resistant *E.faecium* post this organism as third common agents of nosocomial surgical site infection.[3]

3. BLOOD STREAM INFECTIONS

Blood Stream Infections without endocarditis is one of the most common presentation of *Enterococci* and currently the leading cause of nosocomial blood stream infection. *Enterococcus* is the third common organism causing blood stream infections.[23] Frequent causes for blood stream infection are genitourinary and gastrointestinal tract whereas for nosocomial blood stream infection causes are intravascular or urinary catheters. Enterococcal blood stream infection is common in chronically debilitated patients receiving antibiotics and serious underlying conditions. blood stream infection due to *E.faecium* have worst prognosis than *E.faecalis* because they are more resistant to antibiotics and are difficult to treat.[24]

4. ENDOCARDITIS

Enterococci are second or third most common cause of endocarditis after *Staphylococcus* and *Streptococcus* accounting for 5-20% of endocarditis. *E.faecalis* is more common , it affects both native and prosthetic valves and both community and nosocomial associated endocarditis. Sources of infection are genitourinary and gastrointestinal tract, procedures like cystoscopy, trans rectal prostatic biopsy, liver biopsy, surgeries like caesarean section & prostatectomy. Common complications are heart failure and embolisation (27-43%) and mortality ranges from 11- 35%.[25]

5. MENINGITIS

Enterococci are rare causes of meningitis, accounts for 0.3% to 4% of meningitidis cases.[26] It presents as spontaneous and postoperative meningitis. *E. faecalis* is the most common species followed by *E. faecium, E. gallinarum, E. avium,* and *E.casseliflavus*. Spontaneous meningitis in adults is a community-associated infection presents with severe comorbidities, such as diabetes, chronic renal failure, pulmonary or cardiovascular disease, immunosuppression, malignancies, transplantation, and splenectomy. In children, spontaneous meningitis has been reported and is associated with central nervous system pathology like neural tube defects and hydrocephalus, prematurity, recent surgery, or congenital heart disease.[27] Postoperative meningitis is a hospital-associated infection, that presents with shunt devices. Complications of Enterococcal meningitis are hydrocephalus, brain abscesses and stroke. Overall mortality rate is approximately 20%.

6. NEONATAL INFECTIONS

Enterococci are part of the normal adult vaginal flora and infections can be acquired by neonates during delivery. *Enterococcus* accounts for approximately 6% of late-onset sepsis, 5% of pneumonias, 9% of surgical site infections, 10% of blood stream infections, and 17% of UTIs in neonatal units.[28] Risk factors are prolonged hospital stay, low birthweight, prematurity, prior antibiotic therapy, endovascular devices, nasogastric tubes, and several invasive procedures.[3] Outbreaks of VRE causing sepsis in neonates have also been well documented in different parts of the world.

7. SKIN AND SOFT TISSUE AND OTHER INFECTIONS

Decubitus and diabetic foot ulcers are the common lesions associated with *Enterococcus* and in some cases, the organisms have been associated with osteomyelitis.[29] In diabetic wound infection there is transfer of the v*an A g*ene cluster (encoding proteins necessary for vancomycin resistance) from vancomycin -resistant *E. faecalis* to *Staphylococcus aureus.*[30] *Enterococci* are rare causes of soft tissue abscesses such as liver, lung, brain abscesses.[31] and breast abscess caused by *E.faecium* in a patient hospitalized in the critical care unit.

SPECIES OF *ENTEROCOCCI*

Enterococcus species can be classified into five groups as proposed by Facklam and Collins. Classification was based on acid production from mannitol, sorbose and arginine hydrolysis.[32] Further speciation is based on acid production

from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and tellurite reduction (0.04%), motility and pigment production.

CLASSIFICATION OF ENTEROCOCCI

Group I – consists of 9 species

E.avium,E.raffinosus,E.gilvus,E.pallens,E.saccharolyticus,E.malodoratus, E.pseudoavium, E.divriesei and *E.hawaiiensis.*

They produce acid from mannitol and sorbose, arginine is not hydrolysed.

Group II – consists of 8species

E.faecalis, E..faecium, E.gallinarum, E.casseliflavus, E.mundtii, E.haemoperoxidus, E.sanguinicola, E.ythailandicus.

They produce acid from mannitol and not from sorbose and arginine is hydrolyzed. Majority of the them recovered from human sources.

Group III –consists of 6 species

E.dispar, E.canintestini, E.hirae, E.durans, E.ratti ,and *E.villorum.* They don't produce acid from mannitol and sorbose, but hydrolyse arginine.

Group IV – includes 8 species

E.caccae, E.cecorum, E.aquimarinus, E.phoeniculicola, E.sulfureus, E.asini, E.silesiacus, E.termitis.

They don't produce acid from both mannitol and sorbose and arginine is not hydrolysed.

Group V – consists of 6 species

E.canis, E.columbae, E.moraviensis, E.camelliae, E.hermanniensis, and *E.italicus* They ferment only mannitol producing acid but not sorbose and arginine also not hydrolysed.

BIOFILM PRODUCTION BY *ENTEROCOCCUS*

Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces by an extracellular matrix substances, proteins, polysaccharides and nucleic acids. It is regulated by quorum sensing systems. They are notoriously difficult to eradicate and are the source of many chronic infections. Bacteria in biofilms colonize a wide variety of medical devices like catheters, artificial pacemakers, prosthetic devices. *Enterococci* in biofilms are more highly resistant to antibiotics than planktonically growing *Enterococci*, thus the potential impact of biofilm formation could be significant.[2]

EPIDEMIOLOGY OF BIOFILM FORMATION

The prevalence of biofilm production varies worldwide. In most of the studies conducted *E.faecalis* was more common among biofilm producers than *E.faecium.*

FACTORS INFLUENCING BIOFILM FORMATION

Nutrient contents of the growth medium, such as glucose, serum, availability of iron and CO_2, osmolarity, pH and temperature influence biofilm production.

Other factors are,

1. Esp

2. Gelatinase

3. Multiple genes like fsr locus, gelE, epa, atn..

METHODS TO DETECT BIOFILM FORMATION

1. Tube method
2. Tissue culture plate method
3. Congo red method

TUBE METHOD

Trypticase soy broth with 2% sucrose was inoculated with loopful of microorganisms from overnight culture and incubated for 24 hours at 37^{o}c. The tubes were then decaned and washed thrice with PBS pH 7.2 to remove any nonadherent cells. The tubes were air dried and stained with 0.1% crystal violet for 30 minutes. Excess stains were washed off with deionized water. The tubes were dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lines the sides and bottom of each tube. Tubes were then examined and the amount of biofilm was scored as 0-absent,1-weak, 2-moderate, 3-strong. Ring formation at the liquid interface was not indicative of biofilm formation.

CONGO RED AGAR METHOD

The congo red test is based on the ability of this dye to stain polysaccharides black. For preparing congo red agar, sucrose and congo red stain

(50g/land 0.8g/l) were added to Brain Heart Infusion agar. Enterococcal strains were inoculated on the congo red agar plates and incubated at 37^{o}c for 24 hours. A positive result was indicated by black crystalline morphology. Isolates producing black colonies were considered as strong biofilm producers. Weak biofilm producers produced dark pink colonies. Non–slime producers mostly turned out as dry red colonies.

LABORATORY DIAGNOSIS

COLLECTION, TRANSPORT AND STORAGE OF SPECIMENS

The standard methods of collecting blood, urine, wound swab, pus, bile, catheter tip samples, and other secretions or swab specimens are adequate.

It can be transported on any transport media or on swabs that are kept dry. The clinical samples, should be cultured as early as possible, preferably within 1 hour.

DIRECT EXAMINATION

The direct microscopic examination of Gram-stained smears of normally sterile specimens, such as body fluids from sterile sites, are useful for the diagnosis of Enterococcal infections. Direct examination of certain non sterile specimens may also be informative. Only a presumptive report of the “presence of Gram-positive cocci” can be made, by microscopy itself which cannot differentiate it from most of the other Gram-positive cocci. Culture and appropriate identification techniques should be neccessary for confirmation.[10]

As vancomycin -resistant *Enterococcus* (VRE) pose an important problem worldwide, hospitals should implement surveillance programs for VRE detection. In order to overcome these inherent limitations of culture-based methods of detection, conventional PCR and real-time PCR-based methods have been evaluated for direct detection in clinical and surveillance specimens. The Light Cycler *vanA/vanB* detection assay for screening of VRE in rectal or perianal swabs and commercially available DNA probe for the direct detection of *Enterococcus* in blood cultures.[33]

ISOLATION

Clinical specimens, can be plated onto trypticase soy agar, brain heart infusion agar or any blood agar base containing either 5% sheep, horse or rabbit blood for primary isolation of *Enterococci* . Samples for blood culture are inoculated into conventional blood culture systems. Most of the clinically relevant species grow well at 35^{o} to 37ºC and do not require increased level of CO_2. For specimens obtained from nonsterile sites especially when contaminated with Gram negative bacilli, selective media containing sodium azide, bile salts, antibiotics and esculin, tetrazolium can be used. Recently media with chromogenic substrates are used for isolation of *Enterococci*. However not all *Enterococci* grow on selective media. Use of enrichment broth (Enterococcosel broth- BEA medium with 6µg vancomycin) increases the recovery rate of *Enterococci* especially VRE.

Identification of *Enterococcus* species

The genus identification of Gram positive cocci, catalase negative as "*Enterococcus"* is based on the above said tests in genus description. *Enterococal* species can be identified based on acid production from mannitol and sorbose and hydrolysis of arginine as mentioned above. Further speciation is based on acid production from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and reduction of 0.04% tellurite, motility and pigment production.

CULTURAL CHARACTERISTICS AND MORPHOLOGY

Enterococci are more tolerant to adverse physical and chemical conditions. Growth occurs at temperatures from 10-45^{o}C and survive heating at 60^{o}C for 30 minutes. Growth in 6.5% Nacl is an important characteristic feature to seperate *Enterococci* from group D *Streptococci* which is also Bile Esculin positive. *Enterococci* grow on routine nutrient agar, blood agar and MacConkey agar producing small (1-2mm), circular, translucent, convex colonies with smooth surfaces and entire edges. The ability to grow on bile salt agar medium distinguishes from most *Streptococci* except *S.agalactiae*. On MacConkey agar medium deep pink (magenta colour) colonies are formed due to lactose fermentation. On CHROM agar, they grow as blue coloured isolated pin point colonies. *Enterococci* may produce α or β-hemolysis on agar containing cow, rabbit, horse, or human blood but non-hemolytic on agar containing sheep blood as sheep RBCs are refractory to cytolysin mediated cell lysis. Some strains of *E.durans* are β -hemolytic regardless of the blood agar used. All the other species

are α-hemolytic or non-hemolytic. Strains that appear to produce α-hemolytic are actually non-hemolytic strains producing peroxide. The 'greening' of the agar is due to peroxide action on the blood cells and not due to an α toxin.[34] *E. casseliflavus, E. gilvus, E. mundtii, E.pallens,* and *E. sulfureus* produce yellow pigment on the blood-agar medium. The pigment can be detected by picking up the growth on the white cotton swab and examining the swab for a yellow colour. Chocolate agar, colistin nalidixic acid agar, 5% trypticase soy agar, 5% Brain heart infusion agar would also support growth of *Enterococci* .

SELECTIVE MEDIA FOR *ENTEROCOCCI*

1. Bile–esculin–azide agar
2. Enterococcosel agar /broth (white and surrounded by a black halo)
3. Agar containing tetrazolium salts (centre of the colony is brick-red)
4. Pfizer selective *Enterococcus* agar
5. Cephalexin - aztreonam- arabinose agar.
6. Oxoline esculin agar (OOA).[34]

Identification of *Enterococcus* species by commercial methods

There are several commercially available manual, semi-automated, and automated systems for the identification of *Enterococcus* species. These systems are reliable for the identification of *E.faecalis,* and to a lesser extent, *E.faecium.* Commercial systems now available include: the API 20S, API Rapid ID32 STREP systems, the Crystal Gram-Positive ID, Crystal Rapid Gram-Positive identification system, the Gram-Positive Identification Card of the Vitek system

and the Gram-Positive Identification panel of the Micro Scan Walk/Away system. Approximately 80 percent of all Enterococcal isolates will be accurately identified by any one of these systems except unusual species, should be confirmed by standard reference method.

TYPING METHODS

The increasing documentation of *Enterococcus* as a leading nosocomial pathogen exhibiting resistance to many antibiotics together with the concept of exogenous acquisition of *Enterococcus* infections generated demand for strain and epidemiological studies.

CLASSIC PHENOTYPIC METHODS

1. Bio typing
2. Antibiotyping
3. Serotyping
4. Bacteriocin typing and
5. Bacteriophage typing

Classic phenotypic methods used to investigate the *Enterococcus* species have often failed to adequately discriminate among strains, and they have limited value in epidemiologic studies. However, phenotypic information in association with molecular data can contribute valuable information.[35]

Molecular methods

Based on DNA–DNA hybridization and sequencing of the 16SrRNA genes.

They include:

1. Analysis of whole cell proteins(WCP) profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
2. Vibrational spectroscopic analysis
3. Proton magnetic resonance spectroscopic analysis
4. PCR based typing
    a. Randomly amplified polymorphic DNA (RAPD) analysis
    b. Repetitive element sequence (REP) - PCR assay
5. Analysis of chromosomal restriction profiles by pulsed field gel electrophoresis (PFGE)
6. Field inversion gel electrophoresis (FIGE)/Counter clamped homogenous electric field electrophoresis (CHEF)
7. Multi locus enzyme electrophoresis.
8. Ribotyping
9. Sequencing analysis of the 16S rRNA gene
10. Restriction fragment-length polymorphism analysis of amplified 16S rDNA

Various genes are identified by molecular methods include ddl, van, sodAint, cpn60,efaA,ace,tuf or pEM1225 gene.

FISH (Fluorescent In Situ Hybridization) techniques (PNA FISH) been evaluated for identification of *Enterococci* from positive blood cultures. Analysis of chromosomal restriction profiles by pulsed field gel electrophoresis (PFGE) has been the gold standard method for strain typing and epidemiological outbreaks. It is also useful for the identification of predominant clonal complexes and resistance genes - HLAR high level amino glycosides resistance and VRE-vancomycin resistance. Among the two recent methods MLST- Multilocus sequence typing and MLVA multilocus variable number tandem repeat analysis, MLVA is less expensive and rapid compared to MLST.[34]

ANTIMICROBIAL SUSCEPTIBILITY AND RESISTANCE MECHANISMS [3,5,22,34]

Resistance to several commonly used antimicrobial agents is characteristic of most of the Enterococcal species. Antimicrobial resistance can be classified as either intrinsic or acquired. Intrinsic resistance is inherent or natural chromosomally encoded present in all or most of the *Enterococci*. Specific mechanisms of intrinsic resistance to some antimicrobial agents are associated with particular *Enterococcus* species or group of species. The mechanism of acquired resistance may be due to either mutation in existing DNA or acquisition of new genetic determinants found in plasmids or transposons .

Table 1: INTRINSIC AND ACQUIRED RESISTANCE OF *ENTEROCOCCI*

INTRINSIC RESISTANCE	ACQUIRED RESISTANCE
BLactams (cephalosporins, penicillinase resistant penicillins) Lower concentration of aminoglycosides Fluoroquinolones, Trimethoprim-sulfmetoxazole Clindamycin.	High concentration of β Lactams High concentrations of aminoglycosides Glycopeptides-Vancomycin & teicoplanin Chloramphenicol, Tetracyclines, Macrolides-erythromycin Lincosamide-streptogramins, Rifampin and Nitrofurantoin Fluoroquinolones Recent reports highlight the emergence of resistance to the newer agents like linezolid, daptomycin and quinupristin-dalfopristin.

RESISTANCE TO of β LACTAMS

β-Lactam antibiotics interfere with cell wall synthesis by inhibiting the penicillin binding proteins(PBPs) of susceptible bacteria, thus this class of antibiotics should be the first choice for the treatment of susceptible Enterococcal isolates. Many Enterococcal strains are also tolerant to β-lactams, that is, they are not killed with concentrations of antibiotics up to 16 times higher than the MIC(32ug/ml).

Resistance to penicillins and carbapenems is usually exhibited by *E. faecium* and rarely in *E. faecalis*. The mechanisms of resistance in *E. faecium*

appear to be due to the expression of a resistant *pbp5* allele (*pbp5-R*).[36] which has decreased affinity to ampicillin. β-Lactam resistance in *E.faecalis* is mediated by the production of a β- lactamase enzyme.[37]

Penicillin resistance is directly proportional to the amount of PBP5 produced. Fontana et al showed that the loss of ability of a *E.faecium* to produce PBP5 caused this highly penicillin resistant strain to become hyper susceptible to penicillin. Unlike most Staphylococci, where β-Lactamase production is inducible, β-lactamase production in *Enterococci* is constitutive, low level, and inoculum dependent. Nitrocefin based test is used for reliable detection of β lactamase production in *Enterococci* , whereas disc diffusion and dilution methods are not reliable. *Enterococci* susceptible to penicillin are susceptible to ampicillin and other β lactams. But ampicillin susceptibility does not predict susceptibility to penicillin , separate testing with penicillin is needed. Ampicillin susceptibility can be used to predict imipenem susceptibility in case of *E.faecalis* species.

RESISTANCE TO AMINOGLYCOSIDES

INTRINSIC RESISTANCE

Low level resistance is an inherent property, ribosomally mediated owing to low uptake of drugs. When *Enterococci* are grown in the presence of cell surface inhibitors like penicillin and aminoglycoside uptake is markedly enhanced. For *E.faecalis*, the average MICs for gentamicin and tobramycin are 8-64µg/ml; for streptomycin and kanamycin MICs are 250µg/ml.

ACQUIRED RESISTANCE

Acquired resistance and high level resistance to aminoglycosides are due to conjugative plasmids or transposons. For *Enterococci* β-lactams are not bactericidal, but synergistic and bactericidal effect is usually achieved with the addition of an aminoglycoside. Among the aminoglycosides, gentamicin and streptomycin, are the only two compounds recommended for this synergistic effect in clinical practice. The presence of HLR to both gentamicin and streptomycin abolishes the synergistic effect in clinical practice. HLR to gentamicin is mostly due to the presence of a bifunctional aminoglycoside-modifying enzyme, 2′phosphotransferase 6′acetyltransferase, conferring resistance to gentamicin, tobramycin, netilmicin, kanamycin, amikacin but not streptomycin. HLR to streptomycin can be due to mutations in the 300S ribosomal subunit.[38] and streptomycin adenyl transferase enzyme.[39] Hence gentamicin and streptomycin should be tested individually to predict the resistance to aminoglycosides.

RESISTANCE TO QUINUPRISTIN-DALFOPRISTIN

Quinupristin - dalfopristin is a semisynthetic, parenteral antibiotic which is a combination of streptogramin type A (dalfopristin) and type B (quinupristin). It was the first FDA-approved antibiotic for the treatment of VRE infections particularly in severe vancomycin-resistant *E. faecium* infections. Several combination therapies, Quinupristin – dalfopristin or linezolid is listed by the AHA/IDSA for the treatment of Enterococcal endocarditis patients resistant to β-

lactams, aminoglycosides, and glycopeptides.[40] Nonsusceptibility to Q/D in *Enterococci* may be due to following mechanisms,

1) The macrolide- lincosamide- streptogramin B (MLSB)-type of resistance mediated by the *erm* genes (encoding a 23SrRNA methyl transferase)
2) The presence of the (virginiamycin acetyl transferase) *vatD* and *vatE* genes, which encode acetyltransferases that inactivate streptogramin A.

These genes are carried on plasmids and also confer resistance to the streptogramin and virginiamycin, (antibiotic previously used as a growth promoter in the veterinary industry).

RESISTANCE TO LINEZOLID

Linezolid resistance though rare, appears to be increasing, in patients without previous exposure to the antibiotic. Risk factors for the acquisition of nosocomial linezolid-resistant strains are peripheral vascular disease, solid-organ transplant recipients, total parenteral nutrition, and administration of piperacillin-tazobactam and/or cefepime antibiotics.

Mutations in the central loop of domain V of the 23S rRNA is the common mechanism of resistance. The mutation G2576T (*E. coli* 23S rRNA gene numbering) is commonly found in resistant strains, and other mutations are (G2505A, G2512T, G2513T, C2610G). Non-mutational resistance to linezolid is mediated by the presence of a gene *cfr* (chloramphenicol-florfenicol resistance).

cfr gene may have been transferred from *Enterococci* to a Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.[32]

RESISTANCE TO DAPTOMYCIN

Daptomycin resistance involves changes in genes leads to mutation of membrane protein LiaFSR involved in the cell envelope stress sensing response to antibiotics and a protein of glycerophosphoryl diester phosphodiesterase family. It abolishes the bactericidal activity of these antibiotics against these organisms.

RESISTANCE TO GLYCOPEPTIDES

PHENOTYPIC DESCRIPTION

Nine glycopeptide resistant Enterococcal phenotypes (Van A, B, C, D, E, G, L, M and N) have been described on the basis of the level, inducibility and transferability of resistance to vancomycin and teicoplanin. Van A and Van B are the most prevalent phenotypes and are important clinically.

Table 2: PHENOTYPIC AND GENOTYPIC CLASSIFICATION

PHENOTYPE	GENOTYPE (GENE CLUSTERS)	VANCOMYCIN RESISTANCE	TEICOPLANIN RESISTANCE	TYPE OF RESISTANCE
Van A (common in *E.faecalis* and *E.faecium*)	van A gene cluster	High level resistance MIC-64 ≥1000µg/ml	High level resistance MIC-16-512µg/ml	High level inducible resistance
Van B (common in *E.faecalis* and *E.faecium*)	van B gene cluster	High level resistance MIC 4-512µg/ml	sensitive MIC ≤0.5µg/ml	High level inducible resistance
Van C (*E.gallinarum, E.casseliflavus E.flavescens*)	van C1,C2,C3 gene cluster	Low level resistance MIC-2-32µg/ml	sensitive MIC ≤0.5µg/ml	Low level inducible resistance
Van D	van D gene clusters	Moderate High level resistance MIC 64-256µg/ml		inducible resistance
Van E	van E gene clusters	low level resistance MIC-16µg/ml	sensitive MIC ≤0.5µg/ml	inducible resistance
Van G	van G gene	low level resistance MIC≤16µg/ml	sensitive MIC ≤0.5µg/ml	inducible resistance
Van L	van L gene clusters	low level resistance MIC 8µg/ml	sensitive	inducible resistance
Van M	van M gene	high level resistance MIC-256µg/ml	High level resistance	inducible resistance
Van N	van N gene	low level resistance MIC-16µg/ml	sensitive MIC ≤0.5µg/ml	Constitutive resistance

The genes associated with vancomycin resistance in *Enterococci* encode a ligase responsible for the synthesis of dipeptide D-alanyl -D lactate (high level resistance) or D-alanyl -D serine (low level resistance). This replacement results in almost 1000 fold decrease in vancomycin affinity.

VAN A GLYCOPEPTIDE RESISTANCE

The Van A phenotype, with inducible high-level resistance to vancomycin as well as to teicoplanin, encoded by the *vanA* gene. Transposon 1546 is closely related genetic elements mediate this acquired inducible high-level resistance to vancomycin as well as to teicoplanin. The transfer of high level vancomycin resistance from Staphylococcus aureus via Tn 1546 was described recently. It is distributed in the following species of *Enterococcus* such as *E.faecalis, E.faecium,* E.avium, *E.casseliflavus, E.durans, E.gallinarum, E.mundtii, E.raffinosus and E.sanguinicola*.

VAN B GLYCOPEPTIDE RESISTANCE

The Van B phenotype, with variable (moderate to high) levels of inducible resistance to vancomycin but not typically to teicoplanin is encoded by the *van B (vanB*1 and *vanB*2*)* genes. van B gene cluster consists of genes encoding polypeptides arranged to regulate glycopeptide resistance genes (van R and van S), synthesis of dipeptide D – alanyl –D-lactate (van H and van A) and hydrolysis of precursors of normal peptidoglycan (van X and van Y). The gene is located in plasmid and have been mediated by transposons *Tn1547, Tn1549, Tn5382*. The gene product is "D-alanine D-lactate". It is distributed in *E.faecalis, E.faecium, E.durans* and *E.gallinavum.*

VAN C GLYCOPEPTIDE RESISTANCE

The Van C phenotype, encoded by the *van C* genes, confers non-inducible low-level resistance to vancomycin. This is constitutively expressed and

chromosomal in origin corresponding to intrinsic low level glycopeptide resistance seen in *E. gallinarum, E.casseliflavus* and *E.flavescens*. Variable amounts of D–alanyl –D-alanyl relative to D-serine account for variable vancomycin resistance (Van C1, Van C2,Van C3,Van C4) among Van C phenotype and distributed in *E.gallinarum* (C1) and *E.casseliflavus* (C2-C4).

VAN D GLYCOPEPTIDE RESISTANCE

Van D is located in chromosomes and not transferable to other *Enterococci.* Three clinical isolates of VRE (*E.faecium*)carrying van D resistance trait were first found in Boston .It is found in *E.faecalis, E.faecium, E.avium* and *E.gallinarum.*

VAN E GLYCOPEPTIDE RESISTANCE

Recently described in *E.faecalis* BM 4405 with amino acid sequence identical to Van C (45%), Van B (43%) or Van D (44%).

VAN G GLYCOPEPTIDE RESISTANCE

Initially was found in *E.faecalis* isolates from Australia with low level resistance to vancomycin, yet susceptible to teicoplanin.

VAN L GLYCOPEPTIDE RESISTANCE

First described in *E.faecais* NO6-0364, exhibiting low level resistance to vancomycin (8µg/ml) and sensitive to teicoplanin. Van L gene cluster is similar to Van C operon.

VAN M GLYCOPEPTIDE RESISTANCE

First described in *E.faecium* associated with high level resistance to and sensitive to teicoplanin. Resistance is transferable to *E.faecium* BM4105RF by conjugation.

VAN N GLYCOPEPTIDE RESISTANCE

Associated with low level resistance to vancomycin and sensitive to teicoplanin. Van N gene cluster determined by thermal asymmetric interlaced (TAIL) PCR, was similar to that of the Van C operons. *E.faecium* UCN 71 was the first strain isolated from blood culture. The peptidoglycan ends in D –serine and the resistance operon is constitutively expressed. Van N type resistance is transferable to *E.faecium* by conjugation. Van A and Van B are considered the most clinically relevant phenotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while Van C resistance is an intrinsic characteristic of *E.gallinarum* (*vanC1* genotype) and *E.casseliflavus* (*vanC2* and *vanC3* genotypes) strains.

VANCOMYCIN DEPENDENT *ENTEROCOCCI* (VDE)

VDE are the *Enterococci* that grow only in the presence of vancomycin. First isolated in 1994 from urine of a patient receiving long term therapy with vancomycin. The phenomenon vancomycin dependence is that these *Enterococci* turn off their normal D–alanyl –D-alanyl and grow in the presence of alternate cell wall dipeptide D–alanyl –D-lactate. VDE strains are derived from both *E.faecium* and *E.faecalis*.

VANCOMYCIN RESISTANT ENTEROCOCCI (VRE)

Epidemiology

Vancomycin resistant *Enterococci* were identified as nosocomial pathogen mainly *E.faecalis* and *E.faecium* from England in 1988 by Uttley et al.[33] The presence of VRE was associated with the use of avoparcin-a glycopeptide as a growth promoter in animal feeds and was banned from European countries in 1996.[3] The rates of vancomycin resistance among *E. faecium* clinical isolates in Europe are highest in Greece, the United Kingdom, and Portugal (10–30%), whereas rates in the Scandinavian countries and the Netherlands are <1%and latin American countries <4%. These regional differences are due to the implementation of aggressive infection control policies and higher levels of human antibiotic use in the United States. Emergence of vancomycin -resistant *E. faecium* in different parts are due to a unique hospital associated genetic clade that acquired the genes responsible for vancomycin resistance and other antibiotic resistance determinants.[3]

RISK FACTORS

- Presence of immunosuppression
- Presence of Co-morbid conditions like diabetes, renal failure, high APACHE (Acute physiology and Chronic Health Evaluation) score, malignancy,
- Prolonged hospital stay
- Residence in a long term care facility
- Contact with another colonized /infected patient

- Invasive procedures
- Previous exposure to broad spectrum antibiotics – cephalosporins, vancomycin.
- Use of enteral tube feeding/ sucralfate
- Exposure to contaminated medical equipment
- The most important being exposure to heath care personnel nursing to a known VRE patient.

Colonization and Infection

Colonization of the gastro intestinal tract appears to be the first step in the infective process. In most instances, VRE isolation is from colonized patients than infected individual. Colonization usually involves gastrointestinal tract, perineal skin, rarely oral cavity and other sites.[14] VRE infections usually occur in critically ill and debilitated, hospitalized patients. The sites usually involved in VRE infections are bloodstream, intravascular catheters, surgical wounds, prosthetic devices, intra-abdominal sites and urinary tract. It has been reported that mortality rate ranging from 46%-70% among patients infected with VRE.[11] The mortality is higher in patients with prolonged VRE infections such as neutropenic patients, liver transplant recipients and seriously ill patients with co-morbid conditions like chronic renal failure. It is difficult to differentiate between colonization and infection, as mostly these infections are polymicrobial in nature and are recovered along with many other pathogens.[1]

Source of infection and transmission of VRE

The source of infection could be Endogenous – patients own Gastrointestinal tract in previously colonized individuals or Exogenous as contaminated environmental surfaces and, medical devices –bed rails, linen, doorknobs, bed pans, stethoscope and blood pressure cuffs.[8] Contaminated food products may be a reservoir in non hospitalized individuals.[11] The most common mode of transmission is through the contaminated hands of healthcare workers in nosocomial VRE infections.[11] and less commonly with contaminated equipment and contaminated surfaces.

PREVENTION AND CONTROL

The first guidelines for the control of VRE in hospitals was first published in 1994 by HICPAC. CDC's Hospital Infection control practices advisory committee has established certain guidelines and recommendations for prevention of VRE spread.

1. Prudent use of vancomycin - inappropriate use of vancomycin is a risk factor for VRE colonization and infection and also emergence of vancomycin resistant *Staphylococci*. The medical staff should be educated about the appropriate or acceptable use of vancomycin (MRSA treatment, Severe antibiotic associated colitis as a second line agent, major surgical procedures involving implantation of prosthetic devices).
2. Education of the personnel - among all health care workers and patient care givers about the epidemiology and impact of VRE infections.

3. Implementation of surveillance procedures (feces cultures) for early detection of VRE colonization .
4. Infection control procedures aiming to limit cross contamination isolation of known VRE patients and colonizers, strict adherence to hand washing.

To minimize the nosocomial transmission of VRE, hospitals must use a multidisciplinary approach that requires participation by a variety of departments and personnel.

TREATMENT

The suggested therapeutic options for serious VRE infections are

1. Combination therapy with high dose of cell wall active agents (penicillin, ampicillin) and an aminoglycoside (if there is no acquired resistance observed for the agents).[3]
2. The use of ciprofloxacin and other quinolones are limited to the treatment of UTIs. Chloramphenical retains its *in vitro* activity against many strains of MDR *E.faecium*. Triple therapy (ciprofloxacin–rifampicin-gentamicin) is an excellent choice for sterilizing vegetations.
3. Linezolid – It is an FDA approved drug for treatment of VRE infections caused by both *E.faecalis& E.faecium* . It is a bacteriostatic drug belongs to oxazolindones. However it plays a crucial role in the treatment of meningitis and other CNS infections.

4. Quinupristin-Dalfopristin – parenteral semisynthetic streptogramin type A and B and is FDA approved. It is also a bacteriostatic. It is active against *E.faecium* only and not active in *E.faecalis*
5. Daptomycin- it is an acidic lipopeptide active against both *E.faecalis* and *E.faecium* but it is not FDA approved. It is used as an alternative in situations of therapeutic failure of the commonly used agents.
6. Oritavancin is an investigational semisynthetic glycopeptide with potent *in vitro* activity against VRE.

ANTIBIOTIC THERAPY FOR MDR ENTEROCOCCI

Success of ampicillin or vancomycin for *E.faecalis* endocarditis with high level resistance to aminoglycosides have been reported. Tetracycline, chloramphenicol and fluoroquinolones are often used in combination therapy for infections due to MDR *Enterococci*. American heart association (ASA) lists Quinupristin-dalfopristin, linezolid as an option for treating MDR Enterococcal endocarditis. Telavancin, tigecycline, daptomycin are the approved agents for the treatment of complicated skin infections caused by *Enterococcus* sensitive to vancomycin. Rifampin alone has very limited usefulness in the treatment of Enterococcal infections because of its poor bactericidal activity and the presence of subpopulations of resistant bacteria both *in vitro* and *vivo*. Fosfomycin has activity against *Enterococci*, but rapid emergence of resistance limits its usefulness as a single agent.

Materials and methods

MATERIALS AND METHODS

Study Design: Cross sectional study

The present study was conducted in Institute of Microbiology,

Rajiv Gandhi Government General Hospital, Madras Medical College, Chennai, India.

Study Population:

Patients attending outpatient department and inpatients of Rajiv Gandhi Government General Hospital, Madras Medical College, Chennai.

Study Period: 1 year (April 2016- March 2017)

ETHICAL CLEARANCE

Institutional Ethical committee clearance was obtained for the study under the clearance number 22042016.

INCLUSION CRITERIA

Patients aged >18 years

All nonduplicate clinically significant *Enterococcus* isolates from samples such as urine, pus, blood, wound swab, catheter tip and other body fluids were included.

EXCLUSION CRITERIA

Patients aged <18 years and repetitive isolates were excluded.

A total of 100 *Enterococcus* isolates were recovered from various clinical specimens such as urine, blood, pus, wound swab, sterile body fluids and central venous catheters.

METHODOLOGY

Collection and Processing of samples:

All the clinical specimens such as urine, blood, wound exudates, pus, central venous catheter and sterile body fluids from both inpatients and outpatients were collected as per standard protocol.[2] Microscopic examination of the direct Gram stained smears of the specimens were done to look for cellular inflammatory response and presence of microorganisms.

CULTURE[41]

The urine samples were inoculated on cystine lactose electrolyte deficient agar (CLED) and 5% sheep blood agar medium by standard calibrated loop method to perform semiquantitative culture. A biphasic media using brain heart infusion broth and agar was used for isolating *Enterococci* from blood samples. The pus samples and other body fluids from normally sterile sites were inoculated on 5% sheep blood agar and MacConkey agar. Central venous catheter tips were processed by semiquantitative culture as per standard protocol. The inoculated media were incubated at 37°C overnight and observed for growth.

COLONY CHARACTERISTICS

BLOOD AGAR: Small, 1-2 mm, circular translucent convex colonies with regular margins showing either α or non-haemolytic colonies.[22]

MACCONKEY AGAR: Minute, 0.5-1mm, deep pink (magenta) colonies.

CYSTINE LACTOSE ELECTROLYTE DEFICIENT MEDIUM: Small, circular, convex, lactose fermenting colonies are seen. For urine samples, semiquantitative cultures were done using calibrated loop method and only the significant counts (10^5 CFU/ml in clean catch midstream sample, 10^2 CFU/ml in catheterised samples)[42] were processed.

PRELIMINARY IDENTIFICATION OF ENTEROCOCCI

Gram's stain of smear from culture isolates

A smear was prepared from the colonies and Gram stain was done with appropriate controls. The smear was examined for the presence of Gram positive cocci arranged in pairs and short chains.

QUALITY CONTROL

Positive control-*Staphylococcus aureus* ATCC 25923

Negative control-*Escherichia coli* ATCC 25922

CATALASE TEST[2]

Purpose: This test is used to differentiate members of *Enterococcaceae* and *Streptococcaceae* from members of *Staphylococcaceae*. *Enterococci* are catalase negative.

Principle: Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water.

Procedure: A few colonies of culture to be tested were picked from the nutrient agar slope with a sterile, thin glass rod and inserted into 3% hydrogen peroxide solution held in a small, clean test tube.

Interpretation: A positive catalase reaction was indicated by rapid and sustained bubbles or effervescence (nascent oxygen). A few tiny bubbles forming after 20-30 seconds is not considered to be positive test because some bacteria possess enzymes other than catalase that can decompose hydrogen peroxide.

Quality control:

Positive control*: Staphylococcus aureus*

Negative control: *Streptococcus species*

All catalase negative Gram positive cocci in pairs & short chains were further characterized.

BILE ESCULIN TEST[2]

Purpose: This test is used for the presumptive identification of *Enterococci* and some organisms in Streptococcus bovis group. This test differentiates *Enterococci* and group D *Streptococci* from non-group D viridans *Streptococci*.

Principle: Esculin is a glycoside coumarin derivative (6 β Glucoside -7 hydroxy coumarin). For this test esculin is incorporated into the medium containing 4% bile. Esculin hydrolysis in the medium forms glucose and esculitin. Esculitin combines with ferric citrate in the medium to form phenolic iron (black diffusible) complex.

Procedure: Two or three morphologically similar colonies were inoculated on to the slant of the bile esculin medium or streak the surface of a bile esculin plate. Incubate the tube or plate at 35°C for 24–48 hours in an ambient air.

Interpretation: Diffuse blackening of more than half of the slant within 24–48 hours indicates positive test. On plates, black haloes will be observed around isolated colonies and any blackening is considered positive.

Quality control:

Positive control: *Enterococcus faecalis* ATCC 29212

Negative control: *Streptococcus pyogenes* ATCC (19615)-no growth; no colour change or *Escherichia coli* ATCC (25922) - growth; no colour change

HEAT TOLERANCE TEST[41]

Purpose: To differentiate *Enterococci* from other *Streptococcus bovis* and *Streptococcus equinus* group.

Principle: *Enterococci* tolerate temperature of 60^{o}c for 30 minutes.

Procedure: An overnight broth culture of suspected colonies was streaked on blood agar plate. The same broth culture was heated for 30 minutes at 60^{o} C. From this heated broth, another BAP is streaked. Both BAPs were incubated at 37^{o} C for 24 -48 hours.

Interpretation: Growth in both blood agar plates (pre and post heating) was interpreted that, the organism was heat tolerant.

Quality control:

Positive control: *Enterococcus faecalis* (ATCC29212)

Negative control: *Streptococcus bovis* (ATCC 9809)

SALT TOLERANCE TEST[2]

Purpose: This test is used to determine the ability of an organism to grow in high concentrations of salt. It is used to differentiate *Enterococci* from non *Enterococci.*

Principle: This test is useful for the presumptive identification of group D *Enterococci* which have the ability to grow in high salt concentration (6.5% NaCl) in the medium. This test along with bile esculin test distinguishes *Enterococcus* species from the group D *streptococci* (*S.bovis* and *S.equinus*). Group D *streptococci* - bile esculin positive, salt tolerance negative; *Enterococci* – bile esculin and salt tolerance positive. The brain heart infusion broth with 6.5% of NaCl is used as a test medium and bromocresol purple as an indicator for acid production.

Procedure: Two or three colonies were inoculated into 6.5% NaCl broth with or without bromothymol purple indicator and incubated at 35^{o}c in ambient air for 48 hours.

Interpretation: Positive test is indicated by visible turbidity in the broth, with or without colour change (purple to yellow).

Quality control:

Positive control: *Enterococcus faecalis* (ATCC29212) –growth; colour change to yellow.

Negative control: *Streptococcus bovis* (ATCC 9809) – inhibition, as demonstrated by little to no growth; no colour change.

L-pyrrolidonyl β naphthylamide test (PYR TEST)[2]

Principle: This test is used for the identification of Streptococcus pyogenes and *Enterococci* which are PYR test positive. The presence of the enzyme L-pyrrolidonyl arylamidase hydrolyses the substrate α- pyrrolidonyl β naphthylamide and releases free naphthylamide which can be detected by the addition of N, N-methylamino-cinnemaldehyde. This detection reagent couples with the naphthylamide to form a red Schiff base.

Procedure: The colonies were inoculated in PYR broth (α- pyrrolidonyl β naphthylamide) and incubated at 37^{o}C in ambient air for 4 hours. A drop of PYR reagent (0.01% p- dimethylamino-cinnemaldehyde) was added to the broth. Positive test was indicated by the development of cherry red colour within a minute of reagent addition.

Interpretation:

Positive - Bright red colour within 5 minutes.

Negative - No colour change or an orange colour.

Quality control:

Positive control: *Enterococcus* faecalis (ATCC29212) or

Streptococcus pyogenes (ATCC 19615)

Negative control*: Streptococcus agalactiae* (ATCC 10386)

SPECIATION OF *ENTEROCOCCI :*

Enterococci were further identified to species level using Facklam and Collins scheme.

SUGAR FERMENTATION TEST:

For identification of species, 1% of sugars (glucose, arabinose, raffinose, lactose, mannitol, sorbitol) in peptone water with 0.002% bromothymol blue indicator was used. To each tube of sugars, 1-2 drops of 18-24 hours BHI broth culture was added and incubated at 37^{o}C overnight.

Interpretation: Sugar fermentation was indicated by the change of colour from blue to yellow.

ARGININE DIHYDROLASE TEST[2]

Purpose: This test is used to differentiate decarboxylase producing organisms from other.

Principle: This test measures the enzymatic ability (decarboxylase) of an organism to decarboxylate or hydrolyse an aminoacid to form an amine.

Hydrolysis of the amino acid results in an alkaline pH and colour changes to purple.

Method: Moeller's decarboxylase basal broth with 1% arginine along with an aminoacid control were inoculated with the test strain. Both the tubes were overlaid with 4mm sterile liquid paraffin and incubated at 37^{o}C overnight. The colour of the indicator reverting back to original purple colour indicating arginine hydrolysis was considered a positive provided the control tube remains yellow indicating fermentation.

Interpretation: Arginine hydrolysis is positive for Group 2 and Group 3 *Enterococcus* species. Arginine hydrolysis is negative for Group 1, Group 4 and Group 5 *Enterococcus* species.

PYRUVATE UTILIZATION[2]

Principle: This test is used to determine the ability of an organism to utilize pyruvate. This property helps to differentiate *E.faecalis* (positive) and *E.faecium* (negative).

Method: Pyruvate broth with bromothymol blue indicator was highly inoculated with an 18-24 hour culture from 5% sheep blood agar and incubated at 35^{o}C in ambient air for 24-48 hours. Positive test was indicated by colour change from green to yellow whereas negative reaction shows no colour change.

POTASSIUM TELLURITE REDUCTION TEST[2]

Principle: Certain species of *Enterococci* are able to reduce tellurite to metallic tellurium which imparts black colour to the colonies. *E.faecalis* reduces tellurite in contrast to *E.faecium* which does not.

Procedure: 0.04% Tellurite blood agar plates were streaked with overnight nutrient broth culture and incubated at 37^{o} c for 3 days.

Interpretation: Tellurite reduction is considered positive on appearance of black colonies within 3 days.

MOTILITY TEST[2]

Purpose: This test was used to identify *E.gallinarum* and *E.casseliflavus* which exhibit motility. All other *Enterococcus* species are nonmotile.

Hanging drop method: A drop of liquid culture was placed on the cover slip. A hollow ground slide with its concavity encircled by soft petroleum jelly was inverted over the cover slip and quickly turned around. The hanging drop was examined first with a low power objective and then with high power for the presence of motility.

Mannitol motility medium[2]

Purpose: This test is used to identify two characteristics mannitol fermentation and motility.

Method: The motility and fermentation of mannitol was tested by stab inoculating the isolates (including positive and negative control) into the medium and incubated at 37^{o}c overnight detect the motility and acid production from mannitol.

Interpretation: Motility is observed by fanning around the stab area and mannitol fermentation can be detected by colour change of the medium to yellow.

Quality control:

Positive control: *Escherichia coli* (ATCC 25922)

Negative control*: Staphylococcus aureus* (ATCC 25923)

PIGMENT PRODUCTION

Enterococcus isolates were streaked on blood agar and incubated at 37^{o}C for 24-48 hours. A sterile white cotton swab was used to pick up colonies and observe. Pigment production is detected by yellow or orange tinge on the swab.

***Enterococcus faecalis* – characteristics:**

Gram stain – Gram positive cocci in pairs and short chains, some exhibiting spectacle like arrangement.

Catalase – Negative

Motility – Nonmotile

Colony morphology: Blood agar (5% sheep blood agar): non hemolytic, small, cream coloured, smooth colonies with entire edge. Hemolysis is observed when human, horse or rabbit blood is used.[34]

MacConkey agar: lactose fermenting, magenta coloured colonies.

Bile esculin agar: blackening of the medium due to hydrolysis of esculin in the presence of 40% bile.

Heat tolerance: survives a temperature of 60ºC for 30 minutes.

Salt tolerance: survive a salt concentration of 6.5% NaCl

Mannitol motility medium: nonmotile, ferments mannitol by producing acid.

Pigment production: yellow pigment not produced.

Arginine dihydrolysis: hydrolyses arginine – produces deep purple colour after initial colour change to yellow.

0.04% tellurite agar: produces black coloured colonies.

Carbohydrate utilization (1%): ferments pyruvate, raffinose and sorbitol, but not arabinose.

Enterococcus faecium* - characteristics*:

Gram stain – Gram positive cocci in pairs and short chains, some exhibiting spectacle like arrangement.

Catalase – Negative

Motility – Nonmotile

Colony morphology

Blood agar(5% sheep blood agar): non-hemolytic,

Small, cream coloured, smooth colonies with entire edge. Hemolysis is observed when human, horse or rabbit blood is used.

MacConkey agar: lactose fermenting, majenta coloured colonies.

Bile esculin agar – blackening of the medium- hydrolyses esculin in the presence of 40% bile.

Heat tolerance: survives a temperature of 60ºC for 30 minutes.

Salt tolerance: survives salt concentration of 6.5% NaCl.

Mannitol motility medium: nonmotile, ferments mannitol by producing acid.

Pigment production: pigment not produced.

Arginine dihydrolysis: hydrolyses arginine.

Carbohydrate utilization: ferments arabinose, raffinose is variable.

Table 3: Identification of *Enterococcus* species

IDENTIFICATION	*E.FAECALIS*	*E.FAECIUM*
Pyruvate utilisation	Positive	Negative
Arginine hydrolysis	Positive	Negative
Tellurite reduction	Positive	Negative
Motility	Negative	Negative
Pigmentation	Negative	Negative
Glucose	Fermented	Fermented
Mannitol	Fermented	Fermented
Arabinose	Non Fermented	Fermented
Raffinose	Non Fermented	Variable
Sorbitol	Fermented	Fermented
Sucrose	Fermented	Fermented
Lactose	Fermented	Fermented
Trehalose	Fermented	Fermented
Inulin	Non Fermented	Non Fermented

Other species of *Enterococci* were differentiated based on the following characteristics.[4,14]

Enterococcus species	Arginine hydrolysis	Mannitol motility medium	Sugar fermentation	Motility and pigment production
E.dispar	Hydrolysed	Not fermented non motile	Raffinose,sucrose and pyruvate fermented	-
E.durans	Hydrolysed	Not fermented non motile	Raffinose,sucrose and pyruvate not fermented	-

BIOFILM PRODUCTION[2,43]

There are three different methods for studying biofilm production. The qualitative methods are Tube method and Congo red method. Quantitative method is Microtitre plate method. In this study, Microtitre plate method was performed to demonstrate biofilm production.

Microtitre plate method

Colonies of *Enterococci* isolated from fresh agar plates were inoculated in 10 mL of Trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24 hrs. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µl of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contain inoculated sterile broth. The plates were incubated at 37°C for 24 hours. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Then it is stained by crystal violet (0.1%). Excess stain was removed by using deionized water and the plates were kept for drying.

Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad) at wavelength 570 nm.

Table 4: I**NTERPRETATION OF BIOFILMS BY MICROTITRE PLATE METHOD**

S.NO	OD Value	Biofilm
1	≤ 20D	Weak/non adherent
2	20D – 4OD	Moderate
3	≥ 4OD	Strong

ANTIBIOTIC SUSCEPTIBILITY TESTING[44]

Antibiotic susceptibility testing was performed on Mueller Hinton Agar using Kirby Bauer Disk diffusion method. The bacterial inoculum was prepared by inoculating few colonies in the nutrient broth and incubated for 3-6 hours and the turbidity was adjusted with 0.5 McFarland turbidity standard (1.5×10^8). A sterile swab was dipped in that broth and excess fluid was squeezed out by pressing on the sides of the test tube, and it was streaked on the surface of the agar three times, turning the plate at 60^0C each time to get a lawn culture. Then it was allowed to dry and the antibiotic discs were placed within 15 minutes. The antibiotics tested were penicillin10U, ampicillin10µg, vancomycin 30µg, teicoplanin 30µg, tetracycline 30µg, linezolid 30µg and nitrofurantoin 300µg. High level aminoglycoside resistance was determined using gentamicin120µg and streptomycin 300µg. All the antibiotic discs and materials are procured from Himedia laboratories Pvt.Ltd, Mumbai. The inoculated plates were incubated aerobically at 37^oC overnight for 24 hours. The zone of inhibition around each antibiotic disc was measured using Vernier caliper under reflected light except for vancomycin which was measured using transmitted light. The zone diameters

were interpreted in accordance with the CLSI guidelines 2016 as susceptible, intermediate and resistant.

SCREENING TEST FOR HIGH LEVEL AMINOGLYCOSIDE RESISTANCE ENTEROCOCCI [44] (HLAR)

The Enterococcal isolates were screened for high level resistance to aminoglycosides using the antibiotic discs-high level gentamicin(HLG) on Mueller Hinton agar by standard disc diffusion method as described above using 0.5 McFarland turbidity standard bacterial suspension and incubated at 37^{o}C overnight. The results were interpreted as per CLSI standards as susceptible ≥ 10mm, inconclusive 7-9mm, resistant ≤ 6mm.[44] The isolates showing zone of inhibition 7-9mm were tested again by agar dilution method using brain heart infusion agar (BHI) containing gentamicin 500µg /ml as recommended by CLSI 2016 guidelines.[44] 10µl of the 0.5 McFarland Standard bacterial suspension was spot inoculated onto the agar and incubated aerobically at 35±2ºC for 24-48hrs. Similarly Streptomycin 2000µg /ml was also tested. The results were interpreted as resistant when > 1 colony was observed on the screen agar.

SCREENING OF VANCOMYCIN RESISTANT ENTEROCOCCI

Vancomycin screen agar[44]

BHI agar with vancomycin 6µg/ml was the medium used. Inoculation was done via spotting of 10µl of Enterococcal suspension matching 0.5 McFarland suspension. Alternatively, spotting an area of 10 -15 mm using a swab or streaking the plate is also desired. Plates were incubated at 35^{o}C in the ambient

air for 24 hours. Presence of more than one colony indicates presumptive vancomycin resistance and should be confirmed by determining the Minimum inhibitory concentration (MIC) for vancomycin.[44] *E.faecalis* ATCC 29212 – susceptible and *E.faecalis* ATCC 51299 – resistant.

MINIMAL INHIBITORY CONCENTRATION[44,45]

The minimum inhibitory concentration of vancomycin was done for the Enterococcal isolates grown on vancomycin screen agar. Broth microdilution was performed with pure substance of vancomycin (source-Himedia) as per the CLSI recommended standards. *E.faecalis* ATCC 29212 was used for Quality control.

PREPARATION OF STOCK SOLUTION

Stock solution was prepared using the formula 1000/P*V*C=W

- P= potency of the antibiotic base
- V=volume in ml required
- C=final concentration of solution
- W=weight of the antimicrobial to be dissolved in V.

Preparation of drug concentrations of vancomycin:

The vancomycin drug was dissolved in distilled water and the master dilution was prepared by diluting the required amount of drug in Cation adjusted Mueller- Hinton broth (CAMHB). Serial doubling dilution of the master dilution was performed in Cation adjusted Mueller Hinton broth. Thus the following concentrations of vancomycin 0.5µg/ml, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024µg/ml were prepared using CAMHB.

PROCEDURE

Bacterial suspension was prepared by inoculation of BHI broth. To obtain a final inoculum of 10^5CFU/ml the suspensions were diluted to 1:100 in broth medium. 100 µl of serially diluted vancomycin solution was added to all the rows of wells with a growth control and a drug control. 100µl of control strain was dispensed in the 1st row followed by the isolates in the subsequent rows. To this add 10µl of bacterial inoculum was added to all wells except drug control well and the plates were covered and incubated at 37°C for 24 hours in ambient air with appropriate control strains. The endpoint of MIC is the lowest concentration of drug that inhibits the visible growth of the strain. The MIC results were interpreted as per CLSI 2016 guidelines.[44]

MINIMUMAL INHIBITORY CONCENTRATION FOR TEICOPLANIN

The glycopeptide teicoplanin MIC was also tested by E – strip sourced from Himedia. The MIC results were interpreted according to CLSI 2016 guidelines.[44]

GENOTYPING:

PCR was performed on isolates of vancomycin resistant *Enterococci* (VRE) to detect the presence of vancomycin resistance genes. PCR kit was procured from Helini Biomolecules, Chennai. The DNA was extracted from the Enterococcal isolates by using Helini Pure Fast Bacterial Genomic DNA Mini Spin Prep Kit and subjected to PCR and the gene product viewed by gel electrophoresis. VRE genes were identified by the following protocol.

DNA template preparation:

Fresh broth culture (VRE) of 1 ml were centrifuged at 6000 rpm for 5 minutes, supernatant discarded to get a pellet. The pellet is suspended in 0.2 ml PBS. 20 µl of Lysozyme and 180 µl of lysis buffer, was added and incubated at 37° C for 15 minutes. 400 µl of binding buffer, 5µl of internal control template and 20µl proteinase K added, mixed well by inverting several times. Incubate at 56° C for 15 minutes. To this add 300 µl of ethanol and mixed well. Whole lysate was transferred into Pure Fast spin column and centrifuged at 1000 rpm for 1 minute. 500 µl of wash buffer -1 was added and centrifuged for 30-60 seconds. The same was repeated twice with wash buffer -2. The column was centrifuged for additional 1 minute to remove residual ethanol. The Pure Fast spin column was transferred into a fresh 1.5 ml micro-centrifuge tube. DNA was eluted by adding 100 µl of elution buffer, incubate for 1 minute and centrifuged for 2 minutes. 1 µl of extracted DNA was used for PCR amplification.

PCR Procedure:[5]

The master mix consists of 2U Taq DNA polymerase, 10X Taq reaction buffer, 2mM $Mgcl_{2,}$ 1 µl of 10mM dNTPs mix along with the forward and reverse primers. 10 µl of master mix, 5 µl of the extended Genomic DNA, 5 µl of the primer mix were added constituting a total volume of 20 µl. The primers used at the concentration of 10 picomoles / µl. The van A primers obtained from Helini Biomolecules, were as follows:

Forward primer (5'TGCGCGGAATGGGAAAACGACA-3') and

Reverse primer (5'CAGCCCGAAACAGCCTGCTCAA-3')

The reaction mixture was mixed gently and spin down briefly.

PCR amplification:

Initial denaturation	:	95^{o}C for 5 minutes.
Denaturation	:	94^{o}C for 30 seconds {35 cycles}
Annealing	:	58^{o}C for 30 seconds {35 cycles}
Extension	:	72^{o}C for 30 seconds {35 cycles}
Final extension	:	72^{o}C for 5 minutes

PCR products were subjected to 2% Agarose gel electrophoresis using TAE buffer (2 gm agarose in 100 ml of 1X TAE buffer and melted using micro oven). When the agarose gel temperature was around 60^{o} C, 5µl of Ethidium bromide was added and poured on to the gel cast with comb to cut wells after the agar solidified. 1X TAE buffer was poured into submarine gel tank. The gel platform was carefully placed into the tank, maintaining the tank buffer level 0.5cm above than the gel. PCR samples were loaded after mixing with gel loading dye along with 10 µl HELINI 100bp DNA ladder. Electrophoresis was run at 50V till the dye reaches three fourth distances and observed the bands in UV transilluminator for bands of 450bp size. Gel viewed in UV transilluminator and documented using gel documentation system.

Statistical Analysis: Statistical analysis was done with SPSS. The proportional data of this study was analysed using pearson's chi-square test.

Results

RESULTS

A total of 100 *Enterococcus* species were isolated from various samples including urine, blood, pus, wound and sterile body fluids.

Table 5: AGE AND GENDER DISTRIBUTION AMONG PATIENTS WITH *ENTEROCOCCUS* ISOLATED(n=100)

Age	Male	Female	Number	Percentage
18-20 years	8	3	11	11 %
21-30 years	8	9	17	17 %
31-40 years	8	12	20	20 %
41-50 years	9	11	20	20 %
51-60 years	7	11	18	18 %
61-70 years	2	10	12	12 %
>70 years	0	2	2	2 %
Total	42	58	100	100 %

The mean age of the study group was 42 years and among these females were 58% while males were 42%.

Table 6: SAMPLE DISTRIBUTION AMONG *ENTEROCOCCUS* SPECIES (n=100)

SPECIMEN	NUMBER	PERCENTAGE
Urine	77	77%
Blood	9	9%
Pus	7	7%
Catheter tip	5	5%
Wound swab	1	1%
Bile	1	1%
TOTAL	100	100%

Enterococcus species were most commonly isolated from urine (77%), followed by blood (9%), pus (7%), catheter tip (5%), wound (1%) and bile (1%). Among the 77 urine samples 54 were midstream urine (MSU) and 23 were indewelling urinary catheter specimens. Enterococcal infections were more common among Inpatients (91%) than Outpatients (9%).

Table 7: DISTRIBUTION OF ENTEROCOCCAL ISOLATES AMONG PATIENTS ADMITTED IN VARIOUS WARDS (n=100)

SPECIMEN	ICU	NONICU										
		NS	RTU / NEPH	URO	MED	SUR	CTPO	ORT	STD	SGE	DER	HEP
URINE	3	1	18	12	34	2	2	2	1	-	1	1
BLOOD	5	-	-	-	14	-	-	-	-	-	-	-
PUS	-	1	1	-	-	2	-	3	-	-	-	-
WOUND	-	-	-	-	-	1	-	-	-	-	-	-
CATHETER TIP	1	-	4	-	-	-	-	-	-	-	-	-
BILE	-	-	-	-	-	-	-	-	-	1	-	-
TOTAL	9	2	23	12	38	5	2	5	1	1	1	1

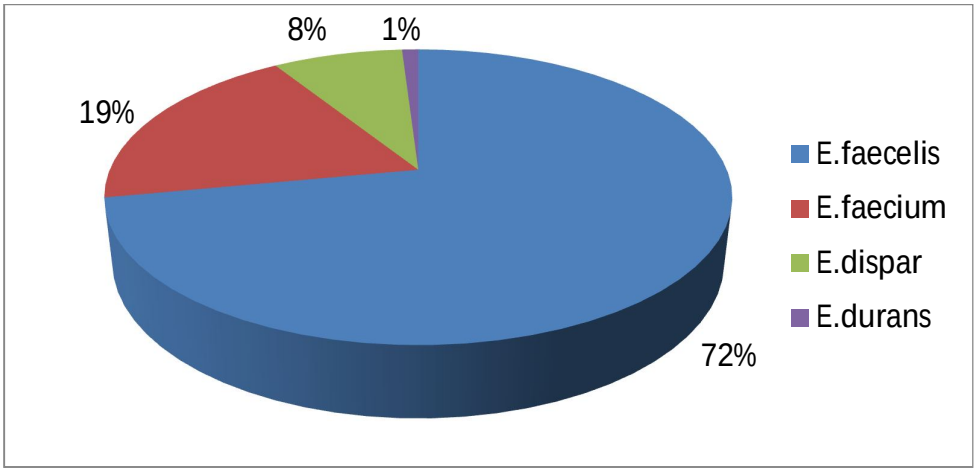
ICU-Intensive care unit, NS- Neurosurgery, RTU- Renal transplant unit, NEPH- Nephrology, URO- Urology, MED- Medicine, SUR- Surgery, CTPO- Cardio thoracic postoperative ward, ORT- Orthopaedics, STD- Sexually transmitted diseases, SGE- Surgical gastro enterology, Der- Dermatology, HEP- Hepatology

Among the 100 *Enterococcus* isolates, 9 were from patients admitted in ICU ward and 91 were from patients admitted in General wards including Medicine ward (38%), Nephrology ward (23%) and Urology ward (12%).

Table 8: DISTRIBUTION OF SPECIES AMONG THE CLINICAL ISOLATES OF *ENTEROCOCCI* (n=100)

SPECIES	NUMBER	PERCENTAGE
E.faecelis	72	72 (%)
E.faecium	19	19 (%)
E.dispar	8	8 (%)
E.durans	1	1 (%)

Figure 1: SPECIES OF ENTEROCOCCI ISOLATED FROM CLINICAL SAMPLES:



Among the *Enterococcus* isolates included in the study, *E.faecalis* (72%) was the most common species followed by *E.faecium* (19%), *E.dispar* (8%), *E.durans* (1%).

Table 9: DISTRIBUTION OF *ENTEROCOCCUS* SPECIES AMONG VARIOUS CLINICAL SPECIMENS(n=100)

	SAMPLES							
***Enterococcus* species**	**URINE (n=77)**			**BLOOD (n=9)**	**PUS (n=7)**	**WOUND SWAB (n=1)**	**CATHETER TIP (n=5)**	**BILE (n=1)**
	MSU	On Foleys Catheter	Total					
E.faecalis	40	16	56	6	5	1	3	1
E.faecium	7	5	12	3	2	-	2	-
E.dispar	6	2	8	-	-	-	-	-
E.durans	1	-	1	-	-	-	-	-
Total	54 (70%)	23 (29%)	77	9	7	1	5	1

Among the *Enterococcus* species 70% of the isolates were from midstream urine, of which *E.faecalis* (52%) and *E.faecium* (9%). 23% were from catheter urine specimens of which *E.faecalis* (21%) and *E.faecium* (6 %).

FIGURE 2: DISTRIBUTION OF *ENTEROCOCCUS* SPECIES AMONG VARIOUS CLINICAL SPECIMENS (n=100)

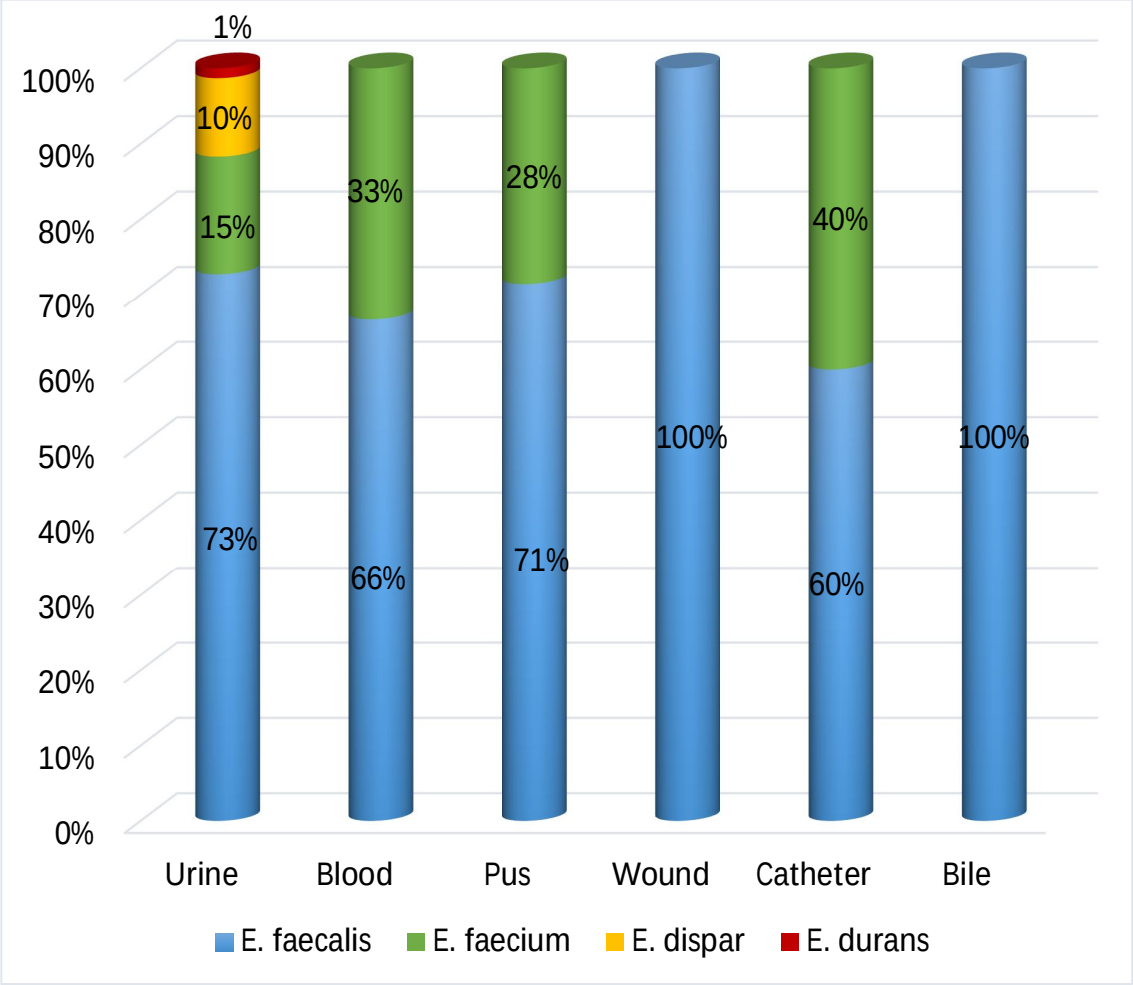


Table 10: CLINICAL DIAGNOSIS IN CULTURE POSITIVE ENTEROCOCCAL INFECTIONS (n=100)

Clinical condition		*ENTEROCOCCUS* SPECIES				
		E.faecalis	***E.faecium***	***E.dispar***	***E.durans***	**Total**
UTI	Uncomplicated (54)	40	7	6	1	77
	CAUTI (23)	16	5	2	-	
Blood stream infections	Blood stream infections (6)	3	3	-	-	14
	IE (3)	3	-	-	-	
	CLABSI (5)	3	2	-	-	
SSI	Intestinal surgeries (3)	3	-	-	-	6
	Orthopaedic Surgeries (3)	1	2	-	-	
ASOM		1	-	-	-	1
Suppurative lymphadenitis		1	-	-	-	1
Biliary tract infection		1	-	-	-	1
Total		72	19	8	1	100

UTI-urinary tract infection, SSI- surgical site infection, CLABSI- catheter line associated blood stream infection, ASOM- acute serous otitis media.

Among 77 patients with urinary tract infections, and 23 patients had CAUTI. Among the 9 patients with blood stream infections, 3 of them had Infective endocarditis and 5 of them had Central line associated blood stream infections.

Table 11: ASSOCIATION BETWEEN *ENTEROCOCCUS* INFECTION AND RISK FACTORS (n=100)

	***ENTEROCOCCUS* SPECIES (n=100)**				
RISK FACTORS	***E.faecalis***	***E.faecium***	***E.dispar***	***E.durans***	**Total**
Foleys catheterization	16	5	2	-	23(23%)
Diabetes	11	5	2	-	18(18%)
Renal disease/ Uropathology	30	12	4	1	47 (47%)
Prolonged hospitalization	13	7	-	1	21(21%)

Among all risk factors renal disease/uropathology accounted 47% followed by urinary catheterization 23% and prolonged hospitalization 21%.

Table 12: ANTIBIOTIC SUSCEPTIBILITY PATTERN: BY KIRBY BAUER DISC DIFFUSION METHOD: FOR URINE SAMPLES (n=77)

Entero coccus	**Pen 30µg**		**Amp 30µg**		**Cip 5µg**		**Tet 30µg**		**HLG 120µg**		**HLS 300µg**		**Van 30µg**		**Nit 30µg**		**Nor 300µg**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
E. faecalis n=55	28 51%	27 49%	23 42%	32 58%	12 22%	43 78%	11 20%	44 80%	19 34%	36 65%	42 76%	13 24%	51 93%	4 7%	45 82%	10 18%	13 24%	42 76%
E. faecium n=13	2 15%	11 85%	2 15%	11 85%	2 15%	11 85%	4 31%	9 69%	4 31%	9 69%	7 54%	6 46%	12 92%	1 8%	10 77%	3 23%	2 15%	11 85%
E. dispar n=8	3 37%	5 62%	3 37%	5 62%	1 12%	7 87%	3 37%	5 62%	3 37%	5 62%	6 75%	2 25%	8 100%	-	7 87%	1 12%	1 12%	7 87%
E. durans n=1	-	1 100%	-	1 100%	1 100%	-	-	1 100%	-	1 100%	-	1 100%	1 100%	-	-	1 100%	-	1 100%
Total	33	44	28	49	16	61	18	59	26	51	55	22	72	5	62	15	15	61
Percent %	43	57	36	64	21	79	23	77	34	66	71	28	93	6	80	19	19	79

The antimicrobial susceptibility pattern reflected that urinary isolates of *E.faecalis* were susceptible to Vancomycin (93%), Nitrofurantoin (82%), Ampicillin (42%) whereas 78% of the isolates were resistant to Ciprofloxacin, 76% to Norfloxacin and 65% to aminoglycosides as tested by HLG. Urinary isolates of *E.faecium* were susceptible to Vancomycin (92%), Nitrofurantoin (77%), HLS (54%) whereas 85% resistant to Ampicillin, Penicillin, Ciprofloxacin & Norfloxacin, 69% to HLG and Tetracycline.

Figure 3: ANTIBIOTIC RESISTANCE PATTERN: BY KIRBY BAUER DISC DIFFUSION METHOD: FOR URINE SAMPLES (n=77)

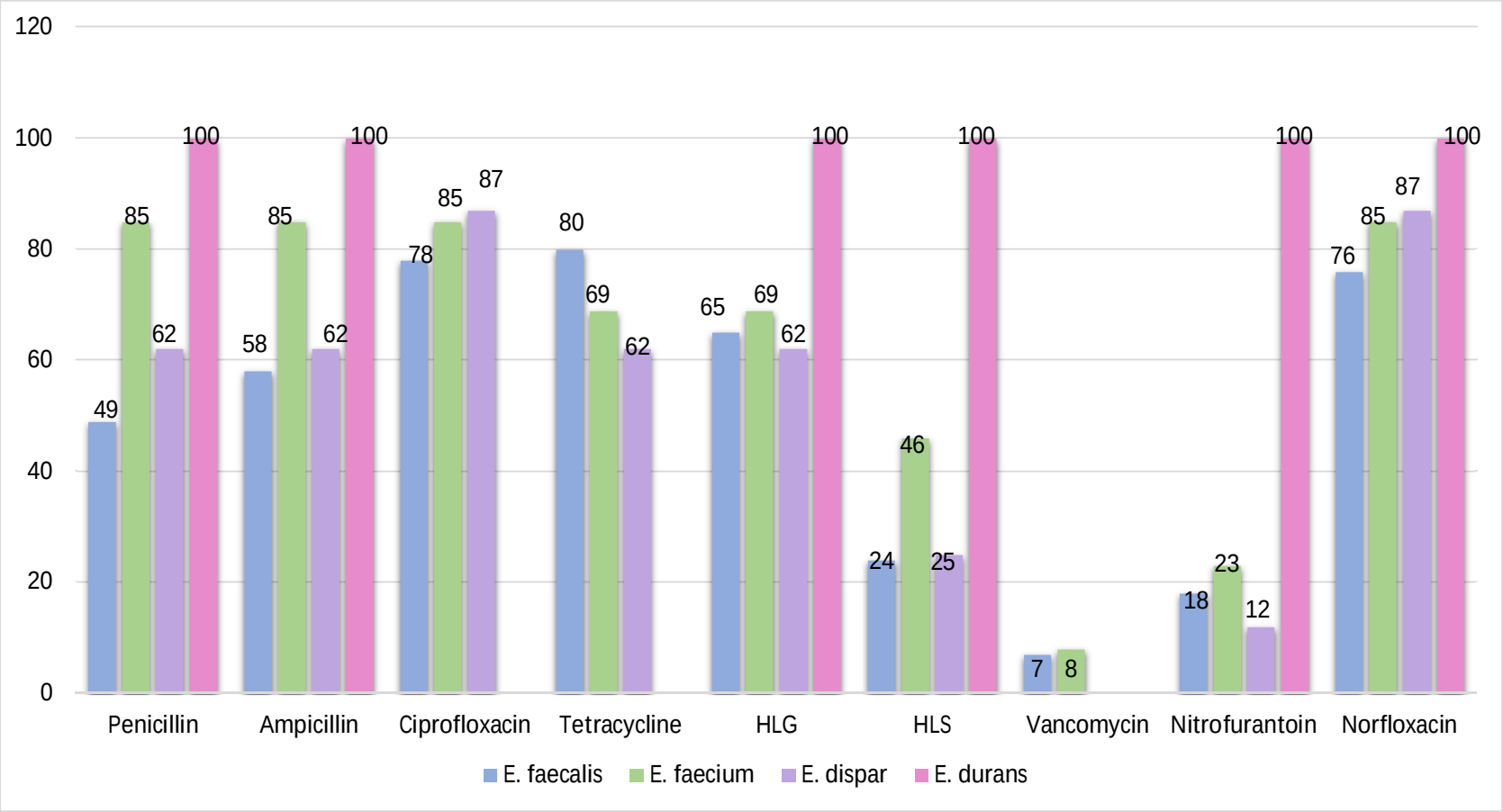


Table 13: ANTIBIOTIC SUSCEPTIBILITY PATTERN: BY KIRBY BAUER DISC DIFFUSION METHOD: FOR SAMPLES BLOOD, CATHETER TIP, PUS, WOUND SWAB, BILE (n=23)

Enterococcus	**Pen 30µg**		**Amp 30µg**		**Cip 5µg**		**Tet 30µg**		**HLG 120µg**		**HLS 300µg**		**Van 30µg**		**Ery 15µg**		**CK (30µg)**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
E.faecalis n=17	4 23%	13 76%	5 29%	12 70%	5 29%	12 70%	7 41%	10 59%	10 59%	7 41%	10 59%	7 41%	12 70%	5 29%	6 35%	11 65%	15 88%	2 12%
E.faecium n=6	-	6 100%	-	6 100%	2 33%	4 66%	2 33%	4 66%	1 17%	5 83%	4 66%	2 33%	5 83%	1 17%	-	6 100%	5 83%	1 17%
Total	4	19	5	18	7	16	9	14	11	12	14	9	17	6	6	17	20	3
Percentage %	17	83	22	78	30	69	39	61	48	52	61	39	74	26	26	74	87	13

Among the *Enterococcus* isolated from blood, pus, catheter tip and wound, *E.faecalis*, 88% were susceptible to chloramphenicol, 70% to vancomycin and 59% to both HLG & HLS whereas 76% were resistant to Penicillin, 70% to Ampicillin & Ciprofloxacin and 59% to tetracycline. Of the *E.faecium* isolated, 83% were susceptible to Vancomycin & Chloramphenicol and 66% to HLS whereas 100% were resistant to Penicillin & Ampicillin and 83% to HLG.

Table 14: HIGH LEVEL AMINOGLYCOSIDE RESISTANCE (HLAR) AMONG *ENTEROCOCCUS* ISOLATES (n=100)

ENTEOCOCCAL SPECIES	RESISTANCE TO HLG ONLY	RESISTANCE TO HLS ONLY	RESISTANCE TO BOTH HLG & HLS	HLAR
E.faecalis (n=72)	29	6	14	49(68%)
E.faecium (n=19)	8	2	6	16(84.2%)
E.dispar (n=8)	3	-	2	5(62.5%)
E.durans (n=1)	-	-	1	1(100%)
Total (100)	40(40%)	8(8%)	23(23%)	71(71%)

HLG-high level gentamicin (120µg), HLS-high level streptomycin (300µg).

The HLAR-high aminoglycoside resistance was observed in 71% of *Enterococcus* isolates and resistance to both agents was observed in 23% whereas monoresistance to gentamicin was observed in 40% and monoresistance to streptomycin was observed in 8%. Resistance shown by *E.faecium* (84.2%) which was more than *E.faecalis* (68%).

Table15: HLG and HLS resistance among *Enterococcus* species (n=100)

High level aminoglycosides		HLS		Total
		R	S	
HLG	R	23	40	63
	S	8	29	37
Total		31	69	100

There was no statistically significant association between HLGR and HLSR with the P value of 0.120 (Pearson chi-square test).

All the Enterococcal isolates which tested resistance to vancomycin in disc diffusion test were inoculated on vancomycin screen agar and subjected to Microbroth dilution test for confirmation of vancomycin resistance. Eight (6 *E.faecalis* and 2 *E.faecium*) out of 10 isolates showed resistance to vancomycin by both the methods.

Table 16: Comparison of Vancomycin Screen Agar and Microbroth dilution test: (n=10)

		Microbroth Dilution		Total
		Susceptible	Resistant	
Vancomycin Screen Agar	Susceptible	2	0	2
	Resistant	0	8	8
Total		2	8	10

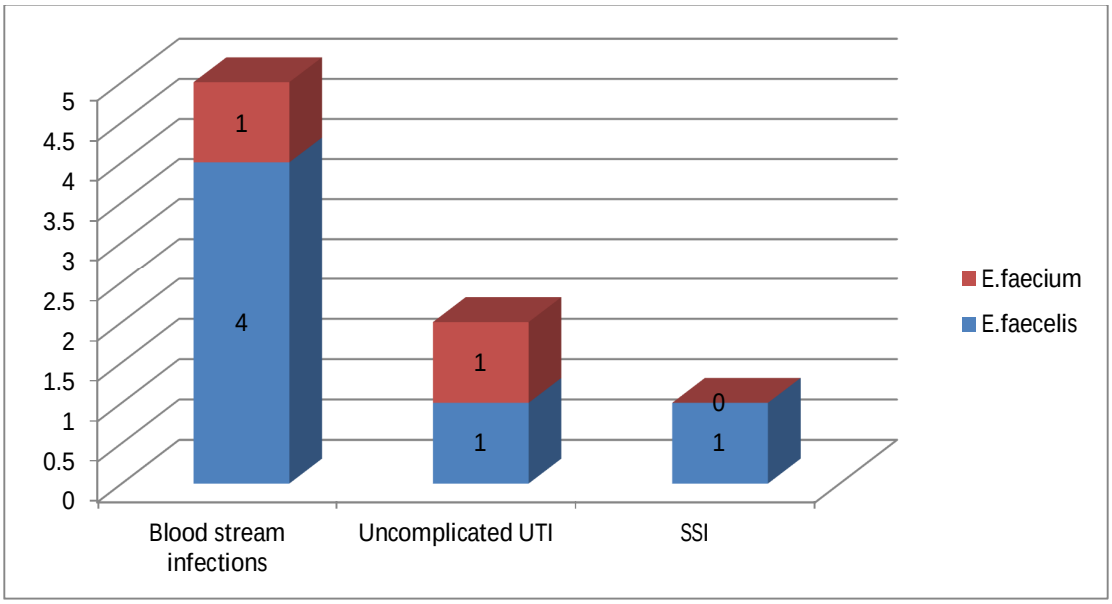
In this study vancomycin resistance detected by vancomycin screen agar was 100% concordance with standard Microbroth dilution method. All the eight VRE isolates confirmed by standard MIC method showed high level resistance to vancomycin (≥64µg/ml).

Table 17: Profile of Vancomycin Resistance *Enterococcus* :(n=8)

VRE species	Blood stream infections	Uncomplicated UTI	SSI	Total
E.faecalis	4*	1	1	6 (6%)
E.faecium	1	1	-	2(2%)
Total	5	2	1	8(8%)
*Out of 4, 1 was CLABSI and 2 were IE, 1 was blood stream infection with unknown primary.				

UTI- Urinary Tract Infection; SSI-Surgical site infection; CLABSI-Central line associated blood stream infection.

Figure 4: Profile of Vancomycin Resistance *Enterococcus*



Classification of VRE by Phenotypic and genotypic method:

Prevalence of VRE was 8%. All the VRE isolates were phenotypically identified as Van A type, which were further subjected to PCR for detection of genotype. All the VRE isolates were of van A genotype and there was 100% correlation between phenotypes and genotypes.

The VRE isolates were tested with linezolid and Quinupristin /Dalfopristin by Kirby Bauer disc diffusion method. The VRE isolates were 100% susceptible to linezolid and Q/D (*E.faecalis* are inherently resistance to Q/D hence were not tested).

Table 18: BIOFILM FORMATION AMONG *ENTEROCOCCUS* ISOLATES: (n=100)

***ENTEROCOCCUS* SPECIES**	**NON ADHERENT/WEAK**	**MODERATE**	**STRONG**	**TOTAL**
E.faecalis	24	37	11	72
E.faecium	5	9	5	19
E.dispar	4	3	1	8
E.durans	1	-	-	1
TOTAL	33(33%)	50(50%)	17(17%)	100

Among all Enterococcal isolates, biofilm formation was exhibited by 67% and among them strong biofilm formation was only 17%.

Table 19: Distribution of biofilm producing *Enterococci* in patients with device associated infections and other infections (n=100)

Type of Infection	**BIOFILM**			**TOTAL**
	Strong	**Moderate**	**Weak/non-adherent**	
CAUTI(n=23)	7 (30%)	13 (56%)	3 (13%)	23
CLABSI(n=5)	2 (40%)	1 (20%)	2 (40%)	5
OTHER INFECTIONS*(n=72)	10 (13%)	36 (50%)	26 (36%)	72
TOTAL	19	50	31	100
*Infections not associated with devices				

There was no statistically significant association between biofilm formation and device associated enterococcal infection with the P value of 0.076 (Pearson chi-square test)

Figure 5: BIOFILM FORMATION AMONG *ENTEROCOCCUS* ISOLATES (n=100)

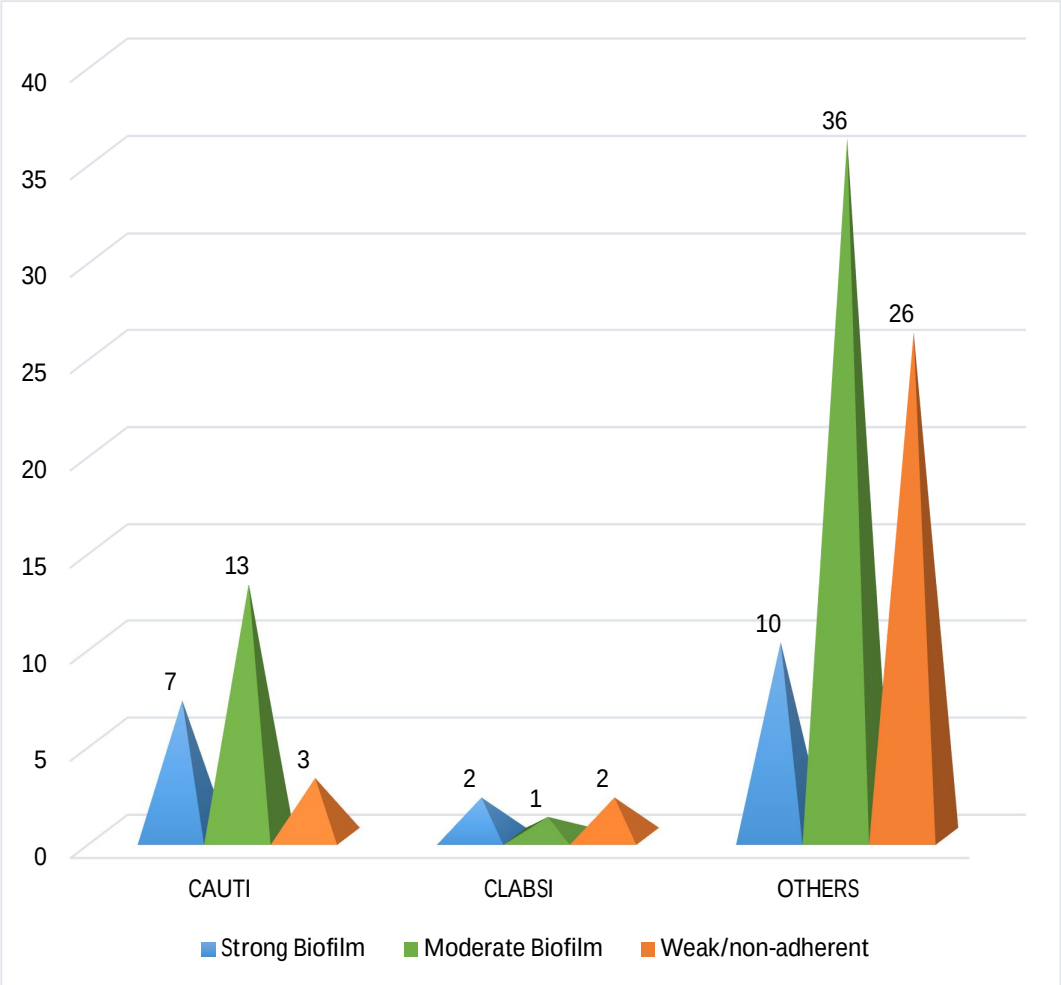


Table 20: CORRELATION BETWEEN BIOFILM FORMATION AND VRE (n=8)

VRE	BIOFILM FORMATION			TOTAL
	STRONG	MODERATE	WEAK/NONADHERENT	
E.faecalis	2	3	1	6
E.faecium	1	1	-	2
	3 (37.5%)	4(50%)	1(12.5%)	8

Of all VRE isolates only 12.5% are non-biofilm producers. There was no statistically significant association between biofilm formation and VRE. (P value - 0.283) by Pearson chi-square test.

Discussion

DISCUSSION

Enterococcus species is one of the most important etiological agents causing health care associated infections and opportunistic infections in immunocompromised patients. *Enterococci* can cause serious life threatening infections like endocarditis, blood stream infections, urinary tract infections and wound infections.[3]Vancomycin Resistant *Enterococci* pose an emerging problem in hospitals worldwide. The prevalence of VRE varies based on geographic location, patient population and antibiotic usage.[46] This study was conducted to assess the prevalence of vancomycin resistant *Enterococci* species isolated from various clinical specimens of patients attending Rajiv Gandhi Government General Hospital.

In this study group, males constituted 58% and female 42%. Mean age of the study population was 42 years. *Enterococcus* isolates were more common among inpatients (91%) than outpatients (9%).

Of the total 100 *Enterococcus* isolates obtained from various clinical samples, distribution of *Enterococcus* species was *E.faecalis (72%) E.faecium* (19%), *E.dispar* (8%) and *E.durans* (1%). This pattern of species distribution is similar to the study conducted in Saudi Arabia by MM.Salem-Bakhit, et al.,[46] in which isolation rate of *E.faecalis* was 69.2% followed by *E.faecium*(11.3%) and other species (6.3%).

In this study *Enterococcus* was commonly isolated from urine (77%) followed by blood (9%), pus (7%), catheter tip (5%), wound swab 1% and bile1%.V.Gupta, et al.,[47] in their study reported a similar finding**,** of *Enterococci* isolated from urine was 49% and blood(5%). In this study, UTI was the most predominant infection (77%) followed by blood stream infection (15%), among which device associated infections constituted 28%(CAUTI 23% & CLABSI 5%). The risk factors associated with Enterococcal infections in this study were underlying renal pathology (47%), indwelling urinary catheters (23%), prolonged hospital stay (21%) and diabetes(18%).

ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *ENTEROCOCCUS* SPECIES

The antimicrobial susceptibility pattern reflected that urinary isolates of *E.faecalis* were susceptible to Vancomycin (93%), Nitrofurantoin (82%), Ampicillin (42%) whereas 78% of the isolates were resistant to Ciprofloxacin, 76% to Norfloxacin and 65% to aminoglycosides as tested by HLG. Urinary isolates of *E.faecium* were susceptible to Vancomycin (92%), Nitrofurantoin (77%), HLS (54%) whereas 85% resistant to Ampicillin, Penicillin, ciprofloxacin & Norfloxacin, 69% to HLG and tetracycline. Among the *Enterococcus* isolated from blood, pus, catheter tip and wound, *E.faecalis*, 88% were susceptible to chloramphenicol, 70% to vancomycin and 59% to both HLG & HLS whereas 76% were resistant to Penicillin, 70% to Ampicillin & Ciprofloxacin and 59% to tetracycline. Of the *E.faecium* isolated, 83% were susceptible to Vancomycin &

Chloramphenicol and 66% to HLS whereas 100% were resistant to Penicillin & Ampicillin and 83% to HLG. In the study conducted by Nita Gangurde, et al.,[48] showed resistance to ampicillin by *E.faecium* (55.1%), *E. faecalis* (41.6%) and resistance to ciprofloxacin by *E.faecium* (86.2%), *E.faecalis* (80.5%).

In the present study, most of the Enterococcal isolates showed resistance to high level gentamicin (63%). Among HLGR Enterococcus isolates, 73.7% was *E.faecium* and 59.7% was *E.faecalis.* These findings were similar to the study is well correlated with the study conducted by L.Triveda, S.Gomathi, et al.,[49] who showed, HLG resistance by *E. faecium* at 67.7% which was higher than *E.faecalis* 46.4% and also HLS resistance by E. *faecium* at 61.3% which was higher than *E.faecalis* 44.3%. *E.faecium* showed more resistance than *E.faecalis* in this study which is similar to the study by L.Triveda, S.Gomathi, et al.,[49] and . In the study conducted by A**.**Hasani et al, Y Sharifi, et al.,[53] showed HLGR of 59.4% and 40.6% among *E.faecalis* and *E.faecium* respectively. In the study conducted by Basabdatta Choudhry, Amit Banik, et al.,[54] HLAR was seen to be higher among *E.faecium* (88.6%) than *E.faecalis* (35.5%)

VANCOMYCIN RESISTANCE *ENTEROCOCCUS*

92% of the *Enterococcus* isolates were susceptible to vancomycin. The prevalence of VRE infection in the present study was 8%. Vancomycin resistant Enterococcal infections were predominant among patients with blood stream infections (50%) and in patients with urinary tract infection (25%). Most common risk factors for VRE in the present study were, prolonged hospital stay and

septicaemia. Among the *Enterococcal faecalis* isolates vancomycin resistance was exhibited in 8.3% compared to *E.faecium* exhibiting higher resistance of 10.5%.A similar result was observed by A. Tripathi, SK Shukla et al[12] reporting VRE prevalence of 7.9% with 10.5% vancomycin resistance among *E.faecium* and 7.9% resistance among *E.faecalis*. All the VRE isolates were 100% resistant to Ampicillin, Penicillin and Ciprofloxacin and 75% & 62.5% to HLS and HLG respectively. 100% of the VRE isolates were susceptible to linezolid and Vancomycin Resistant *E.faecium* isolates were 100% susceptible to Quinupristin/ dalfopristin. Other studies like by V,Gupta,et al[47], and MM Salem- Behkit, et al.[46] reported similar findings that 100% of VRE isolates were susceptible to Linezolid whereas Cressida Auckland, et al.,[55] in their study conducted at London reported the first isolate of Linezolid resistant VRE.

All the VRE isolates exhibit high level resistance to vancomycin (≥ 64µg/ml) and Teicoplanin ≥ 16 µg/ml. PCR results confirmed that all the isolates were of van A genotype which was similar to the study conducted by A. Tripathi et al.[12]

There was 100% concordance of phenotypic and genotypic classification of vancomycin resistance among the VRE isolates in this study, which were observed to be of van A type.

Among all *Enterococcus* isolates biofilm formation was exhibited by 67% of which, 17% were strong biofilm producers. In this study 30% and 56% of the *Enterococcus* isolates from patients with CAUTI were strong and moderate

biofilm producers. 40% and 20% of the isolates from patients with CLABSI were strong and moderate biofilm producers. But this was analyzed to be statistically not significant.

Among VRE isolates strong biofilm formation was observed in 37.5% of isolates. This was similar to the study conducted by A. Tripathi, et al.,[12] were 55.4% were biofilm producers 31.9% were strong biofilm producers.

Summary

SUMMARY

- In this study 100 isolates of *Enterococcus* from various clinical samples namely urine (77%), blood (9%), pus (7%), catheter tip (5%), wound swab (1%) and bile (1%) were speciated and their antimicrobial susceptibility pattern were determined.
- Risk factors associated with *Enterococcus* infections in this study were renal disease (47%), indwelling catheter (23%), prolonged hospital stay (21%) and diabetes mellitus 18%.
- Of the Enterococcus species isolated, *E.faecalis* was observed to be the predominant species (72%), followed by *E.faecium* (19%), E.dispar (8%) and *E.durans*(1%).
- Among the patients with Enterococcal infections, 77 had UTI, of which 29.9% of patients had Catheter associated Urinary tract infection (CAUTI). *E.faecalis* was the predominant species causing uncomplicated UTI (74%) and CAUTI(69.5%) followed by *E.faecium* (12.9% uncomplicated UTI and 21.7% CAUTI).
- Of the 14 patients with Enterococcal blood stream infections, 35.7% patients had CLABSI (60% by E.faecalis and 40% by E.faecium) and 21.4% had Infective Endocarditis. *E.faecalis* was observed to be the causative agent of Infective Endocarditis in this study.
- Urinary isolates of *Enterococcus faecalis*, *Enterococcus faecium* and other species were susceptible to Vancomycin (93%), Nitrofurantoin (82%)

whereas majority of the isolates were resistant to the other drugs. HLG resistance by *Enterococcal faecalis* isolates was 65% and by *Enterococcal faecium* was 69%.

- Isolates of *Enterococcus* from blood and other samples were more susceptible to Chloramphenicol (88% *E.faecalis and* 82% *Enterococcus faecium*) and Vancomycin (70% *E.faecalis and* 88% *Enterococcus faecium*).
- High level aminoglycoside resistance was higher among *E.faecium* (84.2%) than *E.faecalis* (68%).
- The screening tests used for detection of Vancomycin resistance in *Enterococcus* were Vancomycin disc diffusion and Vancomycin screen agar. On comparison of these screening tests to the confirmatory test, microbroth dilution assay, the Vancomycin screen agar was found to be more specific than disc diffusion (100% concordance with Microbroth dilution assay).
- The prevalence of vancomycin resistance among *Enterococci* was 8% in this study.
- The prevalence of vancomycin resistance was higher among *E.faecium* (10.5%) compared to *E.faecalis* (8.3%)
- All the VRE isolates were characterized phenotypically and genotypically to be of van A type.
- The VRE isolates were found to be 100% sensitive to Linezolid and Quinupristin/Dalfopristin.
- The mortality rate among the patients with VRE infections was 25%.

Conclusion

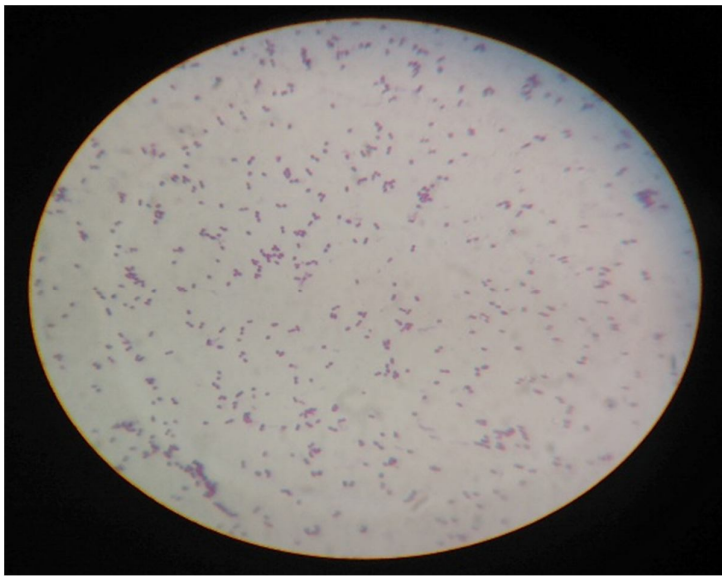
CONCLUSION

Enterococci are emerging as a potential human pathogen causing a wide variety of community acquired and hospital acquired infections that significantly contribute to morbidity and mortality. The emergence of high level aminoglycoside resistance and vancomycin resistance exhibited by *Enterococci* worsen the problem further, leaving fewer therapeutic options for the clinicians in treating the serious life threatening enterococcal infections. The most frequent infection caused by *Enteococci* was studied to be urinary tract infection with *E.faecalis* being the predominant species followed by *E.faecium* though the later showed more resistance.

Biofilm formation was more commonly found in patients with device associated enterococcal infection hence strengthening of adherence to Health care associated infection control bundle care like CAUTI & CLABSI bundle care is recommended. With a prevalence rate of 8% VRE and a mortality rate of 25% in these patients, this study emphasizes the need for conducting frequent surveillance programmes for detection of VRE infections in both community and hospitals and implementation of stringent infection control measures and antibiotic stewardship programs which when followed will decrease the prevalence of VRE among the health care associated infections and thereby significantly reduce morbidity and mortality.

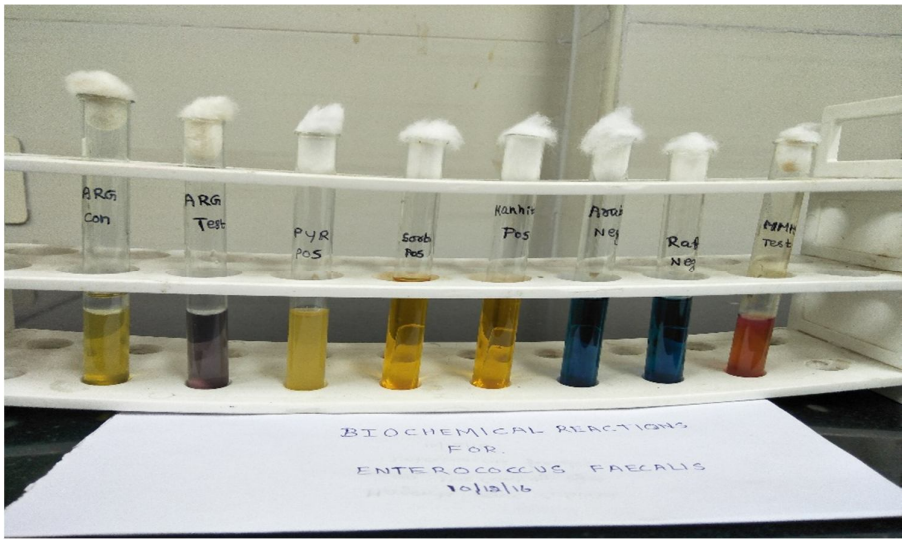
Colour plates

COLOUR PLATES

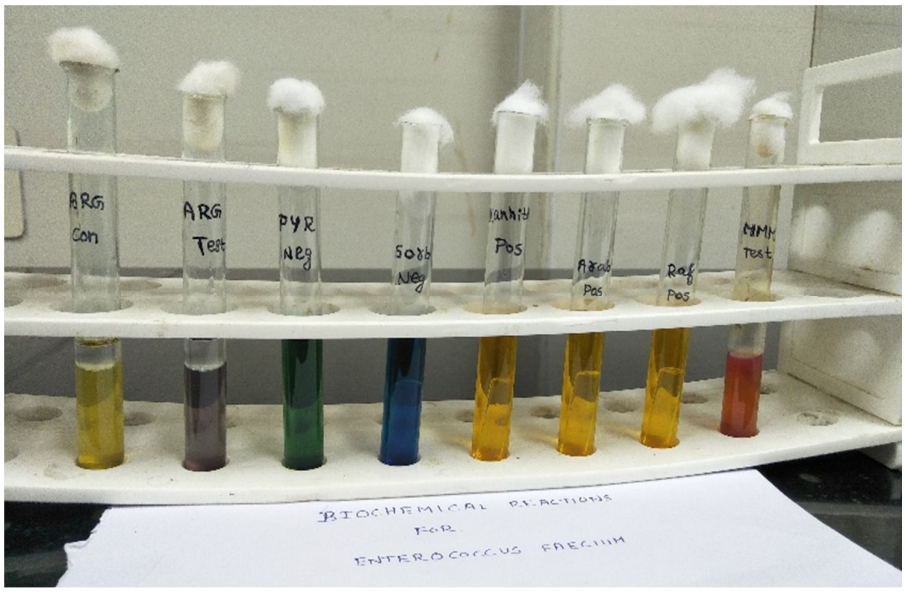


COLOUR PLATE 1: GRAM STAIN OF ENTEROCOCCUS – SPECTACLE EYED APPEARANCE

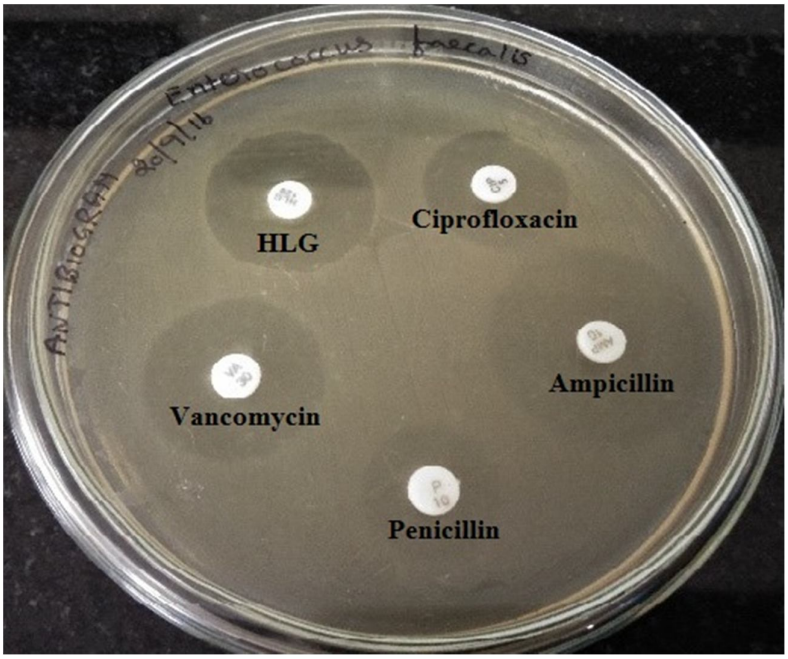
COLOUR PLATE 2: MACCONKEY AGAR PLATE – MAGENTA COLOURED COLONIES



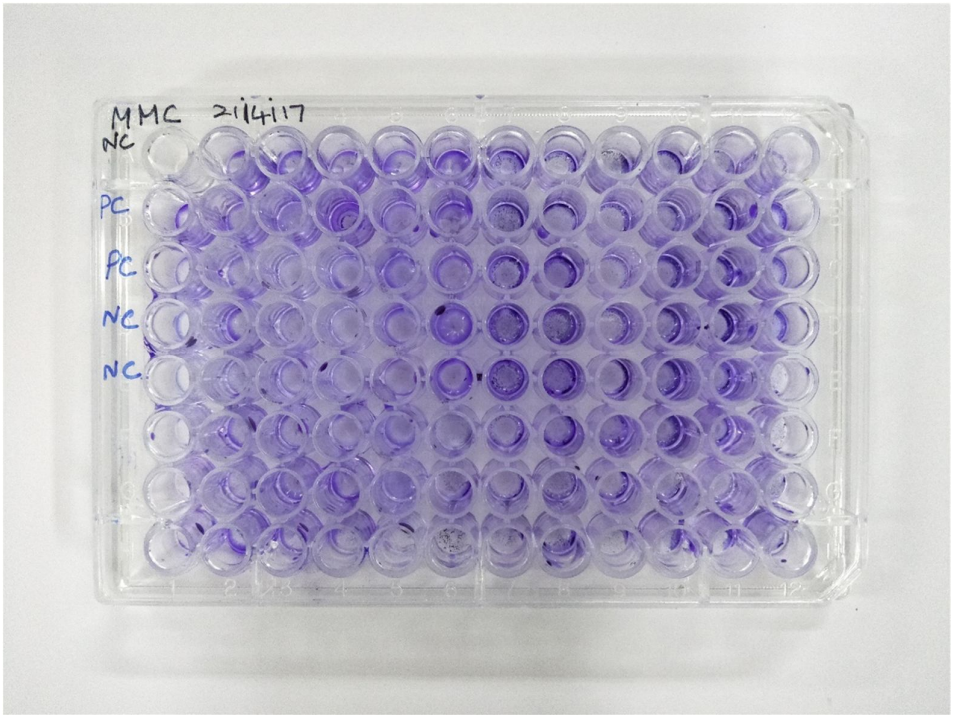
COLOUR PLATE 3: BIOCHEMICAL REACTIONS FOR *ENTEROCOCCUS FAECALIS*



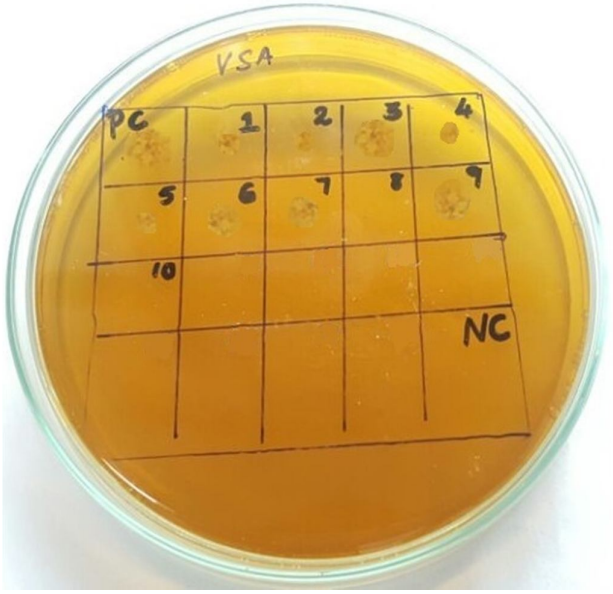
COLOUR PLATE 4: BIOCHEMICAL REACTIONS FOR *ENTEROCOCCUS FAECIUM*



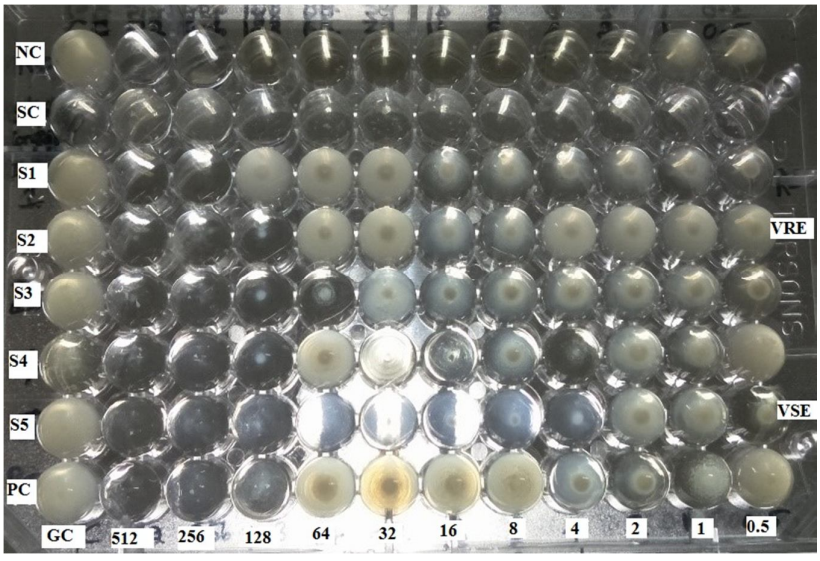
COLOUR PLATE 5: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *ENTEROCOCCUS FAECALIS*



COLOUR PLATE 6: BIOFILM FORMATION BY *ENTEROCOCCUS* ISOLATES

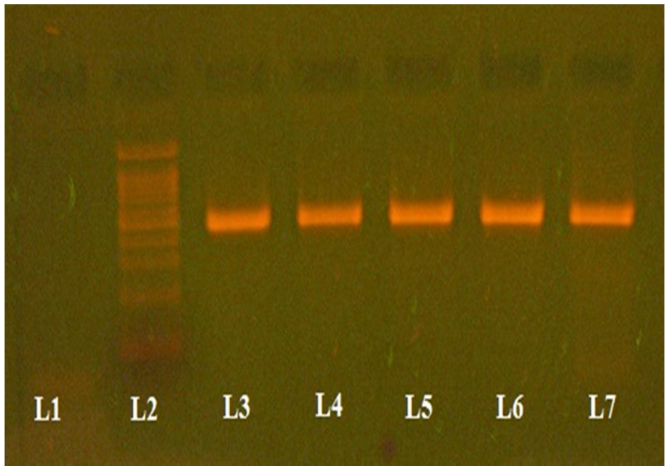


COLOUR PLATE 7: VRE ISOLATES ON VANCOMYCIN SCREEN AGAR

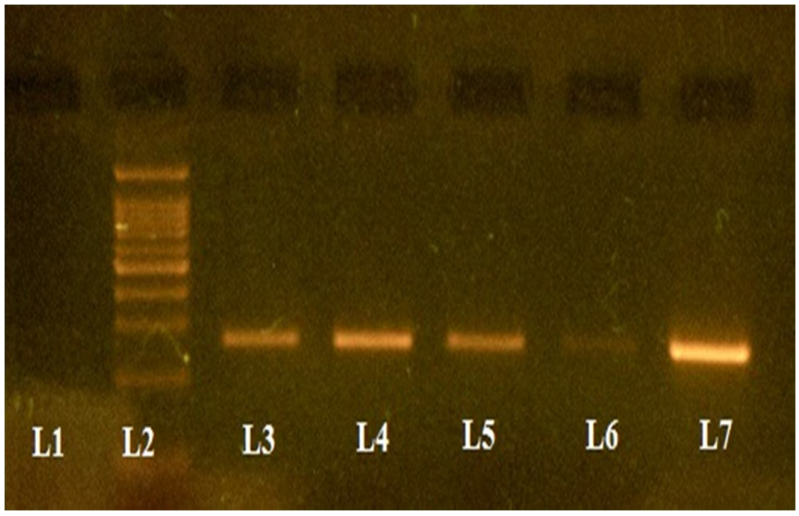


COLOUR PLATE 8: MINIMAL INHIBITORY CONCENTRATION

METHOD OF ENTEROCOCCI FOR VANCOMYCIN



COLOUR PLATE 9: POLYMERASE CHAIN REACTION-Van A GENOTYPE L1–NTC,
L2 - DNA LADDER, L3 – SAMPLE 1, L4-SAMPLE 2, L5 – SAMPLE 3, L6 – SAMPLE 4, L7 – PC



COLOUR PLATE 10: POLYMERASE CHAIN REACTION-
Van A GENOTYPE L1 – NA,
L2 – DNA LADDER, L3 – SAMPLE 5, L4 – SAMPLE 6, L5 - SAMPLE 7, L6 – EMPTY WELL, L7 – SAMPLE 8.

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APPENDIX - I

ABBREVATIONS

AHA	-	American heart association
AS	-	Aggregation substance
ASOM	-	acute serous otitis media
ATCC	-	American type culture collection
BHI	-	Brain heart infusion agar
CAMHB	-	Cation adjusted Mueller- Hinton broth
CAUTI	-	Catheter associated urinary tract infections
CDC	-	The centres for disease control
CFU	-	Colony forming units
CLABSI	-	Catheter line associated blood stream infections
CLABSI	-	Catheter line associated blood stream infection
CLED	-	Cystine lactose electrolyte deficient agar
CLSI	-	Clinical & laboratory standard institute
CTPO	-	Cardio thoracic postoperative ward
Der	-	Dermatology
ELISA	-	Enzyme linked immunosorbent assay
ESP	-	Enterococcal surface protein
FDA	-	Food drug administration
HEP	-	Hepatology
HLAR	-	High level aminoglycoside resistance
HLG	-	High level gentamycin
HLS	-	High level streptomycin
ICU	-	Intensive care unit
ICU	-	Intensive care unit
IDSA	-	Infectious disease society of America
MDR	-	Multidrug resistance
MED	-	Medicine
MLSB	-	Macrolide-lincosamide-streptogramin B

MRSA	-	Methicillin-resistant *Staphylococcus aureus*
MSU	-	Midstream urine
NEPH	-	Nephrology
NNSS	-	National Nosocomial surveillance study
NS	-	Neurosurgery
OD	-	Optical density
OOA	-	Oxoline esculin agar
ORT	-	Orthopaedics
PAI	-	Pathogenicity island
PBPs	-	Penicillin binding proteins
PBS	-	Phosphate buffer saline
PCR	-	Polymerase chain reaction
PYR TEST – L	-	Pyrrolidonyl β naphthylamide test
Q/D	-	Quinupristin – dalfopristin
Q/D	-	Quinupristin - dalfopristin
RBC	-	Red blood corpuscles
RTU	-	Renal transplant unit
SGE	-	Surgical gastro enterology
SPSS	-	Statistical Package for social sciences
SSI	-	Surgical site infection
STD	-	Sexually transmitted diseases
SUR	-	Surgery
TNF	-	Tumor necrosis factor
URO	-	Urology
UTI	-	Urinary tract infection
UTI	-	Urinary tract infection
VRE	-	Vancomycin resistant Enterococci

APPENDIX-II

STAINS, REAGENTS AND MEDIA

Blood agar:

Ingredients

Sterile sheep blood -5 ml

Nutrient agar -100 ml

Autoclave the nutrient agar base at 121º C for 15 minutes. Cool to 45-50º C and add blood with sterile precautions and pour into Petri dish plates.

MacConkey Agar

Ingredients Grams/litre

Peptic digest of animal tissue - 17

Proteose peptone - 3

Lactose - 10

Bile salts - 1.5

Sodium chloride - 5

Neutral red - 0.03

Agar - 15

Final pH at (25º C) 7.1±0.2.

Suspend 51.53 grams in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour into petri dish plates.

Mueller Hinton Agar:

Ingredients

Beef infusion - 300 g/l

Casein acid hydrolysate - 17.50 g/l

Starch -1.50 g/l

Agar -17.00 g/l

Final pH at 25º C 7.4.

Suspend 38 gms in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour 20-25 ml of it into petri dishes of 90 mm diameter to a depth of 4 mm of medium.

Bile Esculin Agar:
Ingredients :
Peptone - 5 gm
Beef extract -3gm
Oxgall(bile) -40gm
Esculin -1gm
Ferric citrate -0.5gm
Agar -15gm
Distilled water -1 L
pH 7.0
heat to dissolve the contents completely, sterilize at autoclave at 121ºC for10 minutes, pour into slants/ petri plates.

6.5% NaCl broth:
Nutrient broth - 1L
NaCl - 6.5gm
Dissolve the contents completely , autoclave at 121ºc for15 min and distribute in tubes.

Brain -Heart infusion agar:
Ingredients:
Agar - 15gm
Bran heart infusion broth -1L
pH 7.4
Dissolve the agar completely by boiling . autoclave at 121ºc for 15 min. cool to about 50ºC and pour into petri dish plates.

Vancomycin Screen agar:
Ingredients:
Agar - 15gm
Brain heart infusion broth - 1 L
Vancomycin - 6mg/L
Prepare Brain heart infusion agar as described above , cool to 50ºC and add Vancomycin 6µg/ml, mix well and pour into petri dish plates.

Cation Adjusted Mueller –Hinton broth: (MHA broth 2) (Himedia lab).
Cation adjusted Mueller- Hinton broth base - 21 gm
Distilled water -1L
Dissolve the contents by boiling and sterilize by autoclaving at 121ºC for 15 min.

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Certificate of Analysis , Quality and Conformity

Material Code : CMS217	**Material Name :** Vancomycin hydrochloride	**Lot No** : 0000231398
AR No.: 090000015950	**Date of Report :** 26.03.2016	**Exp. Date :** May-2018

TEST	SPECIFICATIONS	RESULTS
Appearance		
1 Appearance	White to brown powder	Complies
Solubility		
1 Solubility	Freely soluble in water	Complies
Potency Testing		
1 Antimicrobial Potency Testing	Antimicrobial potency testing of Vancomycin Hydrochloride is carried out by preparing discs of 30 mcg and measuring zone of inhibition of std. organisms on Mueller Hinton Agar (M173) after 18 hrs. at 35-37°C	Complies
Antimicrobial Potency Testing		
1 Zone of inhibition (mm)	17mm - 21mm	20mm
Chemical Analysis		
1 Potency	min.950 µg of vancomycin per mg	Complies
2 FTIR (KBr disc)	Matches with the standard pattern	Complies
3 Assay (HPLC)	>= 80%	84.98%

STATUS OF THE MATERIAL : APPROVED

This is to certify that this lot passes and it confirms to the above mentioned tests and specifications . The information given here is believed to be correct and accurate, however, both the information and products are offered without warranty for any particulars use, other than that specified in the current HiMedia manual or product sheets.

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Pankaj Patil	Sangeeta Kamat	Dr. Santosh Kaul
Microbiologist/Analyst	Dy QC/Dy QA Manager	Quality Assurance Manager

TABLE. 1. ZONE DIAMETER INTERPETIVE STANDARDS FOR ENTEROCOCCUS SPECIES: (CLSI 2016)

Antimicrobial agent	Disk content	Zone diameter		
		Sensitive	Intermediate	Resistant
Penicillin G	10U	≥15	-	≤14
Ampicillin	10µg	≥17	-	≤16
Ciprofloxacin	5µg	≥21	16-20	≤15
Tetracycline	30µg	≥19	15-18	≤14
HLG-high level gentamycin	120µg	≥10	7-9	≤6
HLS-high level streptomycin	300µg	≥10	7-9	≤6
Norfloxacin	10µg	≥17	13-16	≤12
Nitrofurantoin	300µg	≥17	15-16	≤14
Erythromycin	15µg	≥23	14-22	≤13
Chloramphenicol	30µg	≥18	13-17	≤12
Vancomycin	30µg	≥17	15-16	≤14
Linezolid	30µg	≥23	21-22	≤20
Quinupristin-dalfopristin	15µg	≥19	16-18	≤15

TABLE 2: MIC INTERPRETIVE STANDARDS FOR *ENTEROCOCCUS SPECIES (CLSI 2016)*

Antimicrobial agent	MIC µg/ml		
	Sensitive	Intermediate	Resistant
Vancomycin	≤4	8-16	≥32
Teicoplanin	≤8	16	≥32

ANNEXURE-I

PROFORMA

- Name : IP NO:
- Age: Ward:
- Sex:
- Occupation:

  Address:

- Presenting complaints
- Personal history
- Past history
- Clinical diagnosis
- Risk factors
- Prior antibiotic therapy
- Microbiological investigation:
- Microscopic examination of direct gram stained smears:
- Bacterial culture on
- 1.Macconkey agar medium
- 5% sheep blood agar medium

  Phenotypic characterization of Enterococcus species

- Antibiotic sensitivity pattern
- Detection of biofilm formation in Enterococcus species

  Genotypic characterization for the presence of Van A,Van B, Van C .

ANNEXURE - II

CONSENT FORM

STUDY TITLE: "DISTRIBUTION, ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS, WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCI "

I……, hereby give consent to participate in the study conducted by Dr. R. VENNILA, Post graduate at Institute of Microbiology, Madras Medical College, Chennai and to use my personal clinical data and the result of investigations for the purpose of analysis and to study the nature of the disease, I also give consent to give my clinical Specimen (sputum, endotracheal aspirate, bronchial wash, urine, pus, blood) for further investigations. I also learn that there is no additional risk in this study. I also give my consent for my investigator to publish the data in any forum or journal.

Signature/ Thumb impression Place Date
Of the patient/ relative

Patient Name & Address:

Signature of the investigator:

Signature of guide

ANNEXURE-III

INFORMATION SHEET

STUDY TITLE: **"DISTRIBUTION, ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS, WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCI"**

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The aim and objective of the study is to determine the prevalence of enterococcus among various clinical specimens like urine, pus, blood, catheter tip, tracheal aspirates and body fluids. The samples to be inoculated on to the MacConkey agar medium,5% sheep blood agar medium and incubated at 37^0c for 24-48 hours. All plates are to be checked for growth after overnight and after 48 hours. The clinical specimens are subjected to standard biochemical tests. Antimicrobial susceptibility test would be performed on Muller Hinton Agar plates by Kirby – Bauer disc diffusion method. The zone diameters are interpreted in accordance with the CLSI 2015 guidelines. Zone diameter of vancomycin according to CLSI 2015 guidelines is

Sensitive zone - ≥ 17mm
Intermediate zone - 15-16 mm
Resistance zone - ≤ 14mm

Vancomycin resistant Enterococci isolates are then subjected to Microbroth dilution method for determination of Minimum Inhibitory concentration according to CLSI 2015 guidelines. MIC value of vancomycin is

MIC ≤ 4µg/ml - sensitive
MIC = 8-16 µg/ml - Intermediate
MIC ≥ 32 µg/ml - Resistant

Detection of biofilm formation in Enterococcus species by Microtitre plate method. Molecular characterization of Vancomycin resistant Enterococci would be done for the presence of van A, van B or van C genes by Polymerase chain reaction.

MASTER CHART

S. NO	NAME	AGE	SEX	OP/IP	WARD	CLINICAL DIAGNOSIS	RISK FACT	SPECIMEN	ISOLATE	ANTIBIOTIC SUSCEPTIBITY TESTING																BIOFILM
										PEN	AMPI	ERY	CIPRO	TETRA	CHLORA	NOR	NIT	HLG	HLS	VANCO	VRE SCREEN	MIC VAN	VRE GENO	MIC TEI	LZ	RP(Q/D	
1	Selvi	48	F	IP	IMCU	FFE,DM,DCLD	FOLEYS (5)	URINE	E.faecium	R	R	-	R	R	-	R	R	S	R	S							weak
2	Deepika	21	F	IP	RTU	CKD,HT,HEMODIALYSIS	HT	URINE	E.faecalis	R	R	-	R	S	-	R	S	R	S	S	-							weak
3	Venkatesan	30	M	IP	MED	MENINGITIS	FOLEYS (2)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	S	S	-							moderate
4	Nataraj	32	M	IP	RTU	ESRD	FOLEYS (7)	URINE	E.durans	R	R	-	S	R	-	R	R	R	R	S	-							moderate
5	Suresh	39	M	OP	STD	ULCER OVER EXT.GENITALIA	-	URINE	E.faecalis	S	S	-	S	S	-	S	S	R	S	S	-							weak
6	Kamalakannan	63	M	IP	MED	FFE,DM,UTI	-	URINE	E.faecium	R	R	-	R	R	-	R	R	R	R	S	-							weak
7	Saraswathi	38	F	IP	CTPO	POST MVR DONE	FOLEYS (5)	URINE	E.faecalis	S	S	-	S	S	-	R	S	R	S	S	-							moderate
8	Mohan	24	M	IP	SUR	POST APPENDICECTOMY	FOLEYS(3)	WOUND	E.faecalis	R	R	R	R	S	S	-	-	R	R	R	R	>=64	VAN A	>=16	S	R		moderate
9	Subbulakshmi	35	F	IP	MED	HT,HYPOTHYROIDISM	HT	URINE	E.faecalis	S	S	-	S	S	-	S	S	S	S	S	-							moderate
10	Vanaja	38	F	IP	IMCU	IE,SEPTICIMIA	FOL, HOSPITALISED(7)	BLOOD	E.faecalis	R	R	R	R	S	S	-	-	R	R	R	R	>=64	VAN A	>=16	S	R		strong
11	Soniya	23	F	OP	NEPHR	PKD	-	URINE	E.faecalis	R	R	-	S	R	-	R	S	R	R	S	-							moderate
12	Joseph	45	M	IP	MED	HT,DCLD	-	URINE	E.faecalis	R	R	-	S	S	-	S	S	R	S	S	-							moderate
13	Vijayalakshmi	48	F	OP	MED	UTI	-	URINE	E.dispar	S	S	-	R	S	-	R	S	R	S	S	-							weak
14	Radha	48	F	IP	MED	FFE	-	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	S	S	-							weak
15	Ravi	58	M	IP	NEPHR	RF,HT,HYPERTRIGLYCERIDEMIA	HT	URINE	E.faecalis	S	R	-	R	R	-	R	R	R	S	R	S							weak
16	Rajmamary	72	F	IP	NEPHR	DM,DFS,DN	DM	URINE	E.dispar	R	S	-	R	S	-	R	S	R	R	S	-							weak
17	Rajini	20	F	IP	ORTHO	RTA	-	URINE	E.dispar	S	R	-	R	R	-	R	R	R	S	S	-							weak
18	Zahida begam	64	F	IP	NEPHR	DM,DMN,UTI	DM	URINE	E.faecalis	S	S	-	R	R	-	R	S	S	S	S	-							moderate
19	Ganesan	22	M	IP	ORTHO	RTA,# R LEG	FOLEYS(12)	PUS	E.faecalis	R	R	S	R	R	S	-	-	R	S	S	-							weak
20	Gunasundari	62	F	IP	SGE	CHR CAL CHOLYCYSTITIS	-	BILE	E.faecalis	R	R	S	R	R	S	-	-	S	S	S	-							moderate
21	Rekha	29	F	IP	URO	UTI,DM	DM	URINE	E.dispar	R	R	-	R	R	-	R	S	R	R	S	-							strong
22	Jeyaseelan	25	M	IP	IMCU	PUO,THROMBOCYTOPENIA	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-							weak
23	Durai	45	M	IP	SUR	HYDROCELE-OPERATED	FOLEYS(3)	URINE	E.faecalis	R	R	-	R	R	-	S	S	S	S	S	-							weak
24	Gurusamy	24	M	IP	NEPHR	PCKD	-	CATH. TIP	E.faecalis	S	S	R	R	R	R	-	-	S	S	S	-							weak
25	Rathi	35	F	IP	URO	CKD,UTI	HT	URINE	E.faecalis	R	R	-	R	S	-	R	R	R	R	R	R	>=64	VAN A	>=16	S	R		weak
26	Jaya	63	F	IP	ORTHO	DM,#R WRIST	DM	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-							strong
27	Peenam	64	F	IP	URO	CA BLADDER	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	R	S	-							moderate
28	Komathi	35	F	IP	MED	FFE	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-							moderate
29	Pari	37	M	IP	NEPHR	B/L PYLONEPHRITIS	-	URINE	E.faecalis	R	R	-	R	R	-	R	S	S	R	S	-							weak
30	Sivasanmugam	56	M	IP	MED	TPP	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	S	S	S	-							weak
31	Rekha	28	F	IP	NEPHR	ESRD,HEMODIALYSIS	HT,FOLEYS(5)	CATH. TIP	E.faecalis	R	R	R	R	R	S	-	-	S	S	S	-							weak
32	Shanmugam	45	M	IP	SUR	EMERGENCY APPENDICECTOMY	FOLEYS(5)	PUS	E.faecalis	R	R	S	S	S	S	-	-	S	R	S	-							weak
33	Sumathi	32	F	IP	IMCU	RHD,MS	FOLEYS(5)	BLOOD	E.faecalis	R	R	R	R	S	S	-	-	S	S	S	-							weak
34	Yogalakshmi	28	F	IP	URO	PYELONEPHRITIS	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	S	S	S	-							weak

S. NO	NAME	AGE	SEX	OP/IP	WARD	CLINICAL DIAGNOSIS	RISK FACT	SPECIMEN	ISOLATE	ANTIBIOTIC SUSCEPTIBITY TESTING																	BIOFILM
										PEN	AMPI	ERY	CIPRO	TETRA	CHLORA	NOR	NIT	HLG	HLS	VANCO	VRE SCREEN	MIC VAN	VRE GENO	MIC TEI	LZ	RP(Q/D	
35	Murugan	45	M	IP	MED	DM,UTI,DKA	DM	URINE	E.faecium	S	S	-	R	R	-	R	S	S	R	S	-						weak
36	Usha	30	F	IP	URO	RECURRENT UTI	-	URINE	E.faecium	R	R	-	R	R	-	R	S	R	R	S	-						moderate
37	Arun	19	M	IP	MED	FFE	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-						strong
38	Andal	55	F	IP	MED	DM,UTI	DM	URINE	E.faecium	R	R	-	R	S	-	R	R	R	R	S	-						weak
39	Suresh	28	M	IP	NEPHR	UNILATERAL PCKD	-	URINE	E.dispar	R	R	-	S	R	-	R	S	S	S	S	-						moderate
40	Kamala	65	F	IP	MED	AGE	FOLEYS(2)	URINE	E.dispar	S	S	-	R	S	-	S	S	S	S	S	-						weak
41	Rajeshwari	33	F	OP	SKIN	HT,SLE	HT	URINE	E.faecalis	S	S	-	R	R	-	S	S	R	S	S	-						moderate
42	Vijaya	45	F	IP	MED	DM,UTI,PUO	DM	URINE	E.faecalis	S	S	-	S	S	-	R	R	S	R	S	-						moderate
43	Sreesha	19	F	IP	IMCU	HANGING	FOLEYS(7)	URINE	E.faecium	S	S	-	S	S	-	S	S	S	S	S	-						moderate
44	Kamalam	70	F	IP	NEPHR	ORF,HT	FOLEYS(1)	URINE	E.faecalis	R	R	-	R	R	-	S	S	S	S	S	-						moderate
45	Kavitha	33	F	IP	RTU	POST RENAL TRANSPLANT	FOLEYS(5)	URINE	E.faecium	R	R	-	R	R	-	R	S	R	S	S	-						strong
46	Palani	45	M	IP	MED	DCLD,CKD,TB	FOLEYS(2)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	R	S	-						strong
47	Jeeva	55	F	IP	MED	FFE	DM	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-						strong
48	Udayakumar	19	M	IP	CTPO	VSD-OPERATED	FOLEYS(8)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	S	S	-						strong
49	Rasu	55	M	IP	NEPHR	RF,HYPERTRIGLYCERIDEMIA	-	URINE	E.faecium	R	R	-	R	R	-	R	S	R	R	S	-						moderate
50	Suresh	36	M	IP	ORTHO	# L LEG	FOLEYS(7)	PUS	E.faecium	R	R	R	S	S	S	-	-	R	S	S	-						moderate
51	Balakrishnan	60	M	IP	MED	BPH	FOLEYS(1)	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	R	S						strong
52	Sundaramoorthy	33	M	IP	MED	CA BLADDER,RF,DM,HEMODIA	DM	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	R	S	-						strong
53	Komathy	26	F	IP	MED	RA,HYPOTHYROIDISM	-	URINE	E.faecalis	S	S	-	S	R	-	R	S	S	S	S	-						moderate
54	Kamala	60	F	IP	MED	HT,DM,RENAL CALCULUS	DM,HT,FOLEYS(2)	URINE	E.faecalis	R	R	-	R	R	-	R	S	S	S	S	-						strong
55	Govindaraj	44	M	IP	MED	DCLD	FOLEYS(5)	URINE	E.faecium	R	R	-	R	R	-	R	S	S	S	S	-						strong
56	Chandrasekar	45	M	IP	MED	PANCYTOPENIA, NHL	FOLEYS(7)	URINE	E.faecalis	R	R	-	R	R	-	R	S	S	S	S	-						moderate
57	Jamesrupen	75	F	IP	IMCU	HT,META.ENCH, CKD,SEPSIS	HT,FOLEYS(10)	CATH. TIP	E.faecalis	R	R	R	R	S	R	-	-	R	R	R	R	>=64	VAN A	>=16	S	R	strong
58	Kavitha	33	F	IP	RTU	CKD,CADAVER TRANSPLANT	FOLEYS(7)	URINE	E.faecium	R	R	-	S	S	-	R	S	R	S	S	-						strong
59	Rathinammal	55	F	IP	NEPHR	AKI,DM,HT	FOLEYS(2)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	R	S	-						moderate
60	Kavitha	33	F	IP	RTU	CKD,RENAL TRANSPLANT	FOLEYS(7)	CATH. TIP	E.faecalis	R	R	R	S	R	S	-	-	R	S	S	-						strong
61	Merina	19	F	IP	MED	FFE	-	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	R	S	-						moderate
62	Lakshmi	48	F	IP	MED	AML	FOLEYS(1)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	S	S	-						moderate
63	Vignesh	19	M	OP	URO	ANS,NEPHRITIS	-	URINE	E.faecalis	S	S	-	R	S	-	R	S	S	S	S	-						moderate
64	Thilagavathy	45	F	IP	MED	HT,HYPOTHUROIDISM,DEC RF	HT	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	R	S	-						moderate
65	Abraham	20	M	IP	URO	CONGENITAL RENAL AGENESIS	-	URINE	E.faecalis	S	S	-	S	R	-	S	S	S	R	S	-						strong
66	Nagapushmam	58	F	OP	URO	DM,GLOMERULONEPHRITIS	DM	URINE	E.faecalis	S	S	-	R	R	-	R	S	S	S	S	-						moderate
67	Marthandam	55	M	IP	RTU	ACUTE INTES OBST,SSI	-	PUS	E.faecalis	S	S	S	S	R	S	-	-	S	S	S	-						moderate
68	Prathap	20	M	IP	NS	TOF,ASOM,BRAIN ABCESS	-	PUS	E.faecalis	S	S	R	S	R	S	-	-	R	S	S	-						strong

S. NO	NAME	AGE	SEX	OP/IP	WARD	CLINICAL DIAGNOSIS	RISK FACT	SPECIMEN	ISOLATE	ANTIBIOTIC SUSCEPTIBITY TESTING																	BIOFILM
										PEN	AMPI	ERY	CIPRO	TETRA	CHLORA	NOR	NIT	HLG	HLS	VANCO	VRE SCREEN	MIC VAN	VRE GENO	MIC TEI	LZ	RP(Q/D	
69	Jothimani	50	F	IP	MED	DM,HYPOTHYROIDISM	DM	URINE	E.faecalis	S	S	-	R	R	-	R	S	R	S	S	-						moderate
70	Bharathi	42	F	IP	MED	UTI,R PLUERAL EFFUSSION	-	URINE	E.faecalis	R	R	-	S	S	-	S	S	R	S	S	-						moderate
71	Chinnaponnu	63	F	IP	MED	FFE	FOLEYS(1)	URINE	E.faecalis	S	R	-	R	R	-	R	R	R	R	S	-						moderate
72	Raja	52	M	IP	URO	STRITURE URETHRA	-	URINE	E.faecium	R	R	-	R	R	-	R	S	R	S	S	-						strong
73	Kuppammal	54	F	IP	NEPHR	HT,CKD,HEMODIALYSIS	HT	CATH. TIP	E.faecium	R	R	R	R	S	S	-	-	R	R	S	-						moderate
74	Rajkumar	32	M	IP	MED	DM,UTI	DM	URINE	E.faecium	R	R	-	R	R	-	R	S	R	S	R	R	>=64	VAN A	>=16	S	S	moderate
75	Kamalammal	55	F	IP	NEPHR	HT,ESRD	HT	URINE	E.faecium	R	R	-	R	S	-	-	S	R	S	S	-						moderate
76	Rajammal	65	F	IP	MED	CVA,MET.ENCEPHALOPATHY	FOLEYS(7)	BLOOD	E.faecium	R	R	R	R	R	R	-	-	S	S	R	R	>=64	VAN A	>=16	S	S	strong
77	Parvathy	55	F	IP	MED	AGE,FFE	FOLEYS(1)	URINE	E.faecalis	S	R	-	R	R	-	S	S	S	S	S	-						moderate
78	Prabavathy	52	F	OP	MED	HT,ITCHNG OVER EXT GENITAL	-	URINE	E.faecalis	R	R	-	R	R	-	R	S	S	S	S	-						moderate
79	John bosco	46	M	IP	HEPA	CIRROSIS STAGE 3 ,HT	HT	URINE	E.faecalis	R	R	-	S	S	-	S	S	R	S	S	-						moderate
80	Rughda	59	M	IP	SUR	DM,SUPPURATIVE LYMPHADENITIS	DM	PUS	E.faecalis	R	R	S	S	S	S	-	-	R	R	S	-						moderate
81	Rohiya bashan	31	M	IP	NEPHR	AKI	-	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	S	S	-						moderate
82	Rajathi	23	F	IP	RTU	POST RENAL TRANSPLANT	-	URINE	E.dispar	R	R	-	R	R	-	R	S	R	S	S	-						moderate
83	Aadhi	49	M	IP	MED	HT,DCLD	HT,FOLEYS(3)	URINE	E.dispar	R	R	-	R	R	-	R	S	S	S	S	-						moderate
84	Karthikkumar	24	M	IP	RTU	HT,CKD STAGE 3	HT,FOLEYS(5)	URINE	E.faecalis	R	R	-	R	R	-	R	S	R	R	S	-						moderate
85	Chandrakala	45	F	IP	NS	UTI,RTA	-	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	S	S	-						moderate
86	Ponnammal	70	F	IP	NEPHR	DM,HT,CAD	,FOLEYS(3)	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	S	S	-						moderate
87	Sheiknazini	36	F	IP	IMCU	SEPSIS,BA,ACUT R PE	,FOLEYS(5)	BLOOD	E.faecalis	R	S	R	R	R	S	-	-	S	S	S	-						moderate
88	Chinnaponnu	63	F	IP	MED	FFE	FOLEYS(2)	URINE	E.faecalis	R	R	-	R	R	-	R	R	R	S	S	-						weak
89	Sundarrajan	48	F	IP	IMCU	HUS	HT	BLOOD	E.faecium	R	R	R	R	R	S	-	-	R	R	S	-						weak
90	Dineshkumar	19	M	IP	SUR	SUB ACUTE APPENDICITIS	-	URINE	E.faecalis	S	S	-	R	R	-	R	S	S	S	S	-						weak
91	Ganapathi	66	M	OP	URO	HT,BPH	-	URINE	E.faecalis	S	R	-	R	R	-	R	S	R	S	S	-						weak
92	Chinnaponnu	42	F	IP	MED	UTI,PLEURAL EFFISION	-	URINE	E.faecalis	S	R	-	S	R	-	S	S	S	S	S	-						moderate
93	Prathap	40	M	OP	URO	STRITURE URETHRA	-	URINE	E.faecalis	S	S	-	S	R	-	S	S	S	S	S	-						moderate
94	Dinesh	28	M	IP	MED	FFE	FOLEYS(2)	BLOOD	E.faecalis	S	S	S	R	R	S	-	-	S	S	S	-						weak
95	Divinkumar	18	M	IP	ORTHO	# R FOOT	-	PUS	E.faecium	R	R	R	S	R	S	-	-	R	S	S	-						moderate
96	Lakshmi	60	F	IP	IMCU	FFE,SEPSIS	-	BLOOD	E.faecium	R	R	R	R	R	S	-	-	R	S	S	-						weak
97	Arun	19	M	IP	MED	FFE	-	URINE	E.faecalis	S	S	-	R	R	-	S	S	R	S	S	-						weak
98	Jaya	35	F	IP	URO	CKD	-	URINE	E.faecalis	R	R	-	R	S	-	R	S	R	S	S	-						weak
99	Gayathri	28	F	IP	MED	FFE	-	BLOOD	E.faecalis	R	R	R	R	R	S	-	-	S	R	R	R	>=64	VAN A	>=16	S	R	moderate
100	Ameenabee	55	F	IP	MED	MS,MR,IE	FOLEYS(3)	BLOOD	E.faecalis	R	R	R	R	S	S	-	-	S	R	R	R	>=64	VAN A	>=16	S	R	moderate

ANNEXURE-V

LEGENDS FOR MASTER CHART

AGE	-	Acute gastro enteritis
AKI	-	Acute kidney injury
AML	-	Acute myloid leukemia
ANS	-	Acute nephrotic syndrome
ARF	-	Acute renal failure
ASOM	-	Acute suppurative otitis media
BPH	-	Benign prostatic hypertrophy
CAD	-	Coronary artery disease
CKD	-	Chronic kidney disease
CVA	-	Cerebro vascular accident
DCLD	-	Decompensated chronic liver disease
DFS	-	Diabetic foot syndrome
DM	-	Diabetes mellitus
DMN	-	Diabetic nephropathy
ESRD	-	End stage renal disease
F	-	Female
FFE	-	Fever for evaluation
HT	-	Hypertension
IE	-	Infective endocarditis
IP	-	Inpatient
M	-	Male
MIC	-	Minimum inhibitory concentration
MS	-	Mitral stenosis
MVR	-	Mitral valve replacement
NHL	-	Non hodgkins lymphoma
OP	-	Outpatient
PKD	-	Polycystic kidney disease
R	-	Resistant
RA	-	Rheumatoid arthritis

RF	-	Renal failure
RHD	-	Rheumatic heart disease
RTA	-	Road traffic accident
S	-	Susceptible
SLE	-	Systemic Lupus Erythematosis
SSI	-	Surgical site infection
TOF	-	Tetralogy of fallot
TPP	-	Thrombocytopenic purpura
VRE	-	Vancomycin resistant enterococci
VSD	-	Ventricular septal defect

ANNEXURE-VI

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To
Dr.R.Vennila
Post Graduate in M.D. Microbiology
Madras Medical College
Chennai 600 003

Dear Dr.R.Vennila,

The Institutional Ethics Committee has considered your request and approved your study titled **"DISTRIBUTION, ANTIMICROBIAL, SUSCEPTIBILITY PATTERN AND DETECTION OF BIOFILM FORMATION IN ENTEROCOCCUS SPECIES ISOLATED FROM VARIOUS CLINICAL SPECIMENS WITH PHENOTYPIC AND MOLECULAR CHARACTERISATION OF VANCOMYCIN RESISTANT ENTEROCOCCUS " NO. 22042016.**

The following members of Ethics Committee were present in the meeting hold on **05.04.2016** conducted at Madras Medical College, Chennai 3

1.Dr.C.Rajendran, MD.,	:Chairperson
2.Dr.Isaac Christian Moses,MD.Ph.D.Dean(FAC)MMC,Ch-3	:Deputy Chairperson
3.Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3	: Member Secretary
4.Prof.B.Vasanthi,MD., Prof.of Pharmacology.,MMC,Ch-3	: Member
5.Prof.P.Raghumani,MS, Prof. of Surgery,RGGGH,Ch-3	: Member
6. Prof.Md.Ali,MD.,DM.,HOD-MGE, MMC,Ch-3	: Member
7.Prof.Baby Vasumathi, Director, Inst. of O&G,Ch-8	: Member
8.Prof.K.Ramadevi,MD, Director,Inst.of Bio-Chem,MMC,Ch-3	: Member
9.Prof.M.Saraswathi,MD.,Director, Inst.of Path,MMC,Ch-3	: Member
10.Prof.Srinivasagalu,Director,Inst.of Int.Med.,MMC,Ch-3	: Member
11.Tmt.J.Rajalakshmi, JAO,MMC, Ch-3	: Lay Person
12.Thiru S.Govindasamy, BA.,BL,High Court,Chennai	: Lawyer
13.Tmt.Arnold Saulina, MA.,MSW.,	:Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary - Ethics Committee
MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE